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Regulation of c-*myc* through intranuclear localization of its RNA subspecies

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[Running Title]

Localization of c-*myc* RNA subspecies

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Abstract

We used fluorescence *in situ* hybridization (FISH) to detect c-*myc* RNA subspecies in human COLO 320DM tumor cells. Although the FISH procedure removed the majority of RNAs from the nucleolus, c-*myc* RNA continued to be detected in both the nucleoplasm and nucleolus. This finding suggests stable association between c-*myc* RNA and the nucleolus. Nucleolar accumulation of c-*myc* RNA appeared to be temporally regulated by cell cycle progression. Hybridization with exon- and strand-specific RNA probes indicated that the non-protein coding exon 1 plays a novel role in determining the subnuclear localization of c-*myc* RNA. Antisense RNA targeting exon 2 localized only with nucleoplasmic foci, where it might interact with the sense strand. Thus, c-*myc* gene expression may be regulated by intranuclear localization of its RNA.

Keywords; nucleolus; RNA FISH; nuclear structure and function; post-transcriptional gene regulation; c-*myc*

(Introduction)

In addition to ribosomal biogenesis, the nucleolus may be involved in the metabolism of a number of different RNAs. Although the biological significance of nucleolar localization remains unclear, some mRNA species such as *N-myc*, *c-myc* and *myo*D1 mRNA, have been found to accumulate in the nucleoli of several cell lines, and this phenomenon may be involved in regulation of their expression [1-4]. C-*myc* is transcribed from multiple promoters (for a review, see [5]). The major promoter (P2) drives transcription of c-*myc* RNA from exon 1, while another promoter (P3), which is in the intron between exons 1 and 2, drives a transcript that lacks exon 1. In addition, an antisense promoter in the second intron initiates transcription of an antisense strand [6, 7]. Exon 1 does not encode a protein and its function remains to be clarified. Furthermore, the reasons underlying c-*myc* promoter complexity remain unclear.

Here, we detected intranuclear localization of c-*myc* RNA subspecies by non-denaturing fluorescence *in situ* hybridization (RNA-FISH) using exon- and strand-specific RNA probes. We used the human colorectal carcinoma COLO 320DM cell line, in which c-*myc* is amplified *ca*. 64-fold. Overexpression of c-*myc* RNA species can be traced easily in these cells. We observed that two procedures commonly involved in RNA-FISH, i.e., ethanol treatment and hybridization, removed the majority of RNAs from the nucleolus. Following these treatments, c-*myc* RNA was detected in the nucleoli, a finding that suggests the formation of a stable association. We also found evidence suggesting that the protein non-coding exon 1 and promoter complexity might be involved in control of intranuclear localization and post-transcriptional regulation of c-*myc* RNA.

Materials and methods

Cell culture and synchronization. Human COLO 320DM (CCL 220) tumor cells were obtained and maintained as described previously [8]. In this cell line, the c-*myc* oncogene is amplified *ca*. 64-fold and localized to extrachromosomal multiple double minutes (DMs) [8]. Cell-cycle synchronization was performed as described previously [9]. In brief, cells were arrested at S phase with excess thymidine for 17 h and released in fresh medium for 12 h. They were then arrested at the G1/S boundary with aphidicolin for 17 h, released for 9 h and arrested at the prometaphase by nocodazole for 13 h.

Preparation of the probes. A c-*myc* genomic clone containing exon 3 (EX3) was digested with *Cla* I-*Eco* RI and the resulting fragment re-cloned into pGEM4. Exons 1 and 2 (EX1, 2406 to 3589; and EX2; 4669-6530, respectively) were PCR-amplified from the genomic c-*myc* locus (HUMMYCC; GenBank, X00364) of normal human placental DNA. Primer sequences are available upon request. The PCR product was subcloned into pGEM-T (Promega Co. Madison, WI). Plasmid DNA was linearized using an appropriate restriction enzyme and then antisense and sense RNA probes were transcribed using either SP6- or T7-RNA polymerase and DIG-dUTP. RNA probes were ethanol precipitated, dissolved in alkaline hydrolysis buffer (40 mM $NaHCO_3$, 60 mM Na_2CO_3), incubated at 60 ^oC for 23 min and neutralized with acetic acid. The alkaline hydrolysis product (*ca*. 100 to 300 nucleotides) was ethanol precipitated and used for hybridization.

Cytochemical procedures. RNA-FISH was performed as described previously [10, 11]. Cells were washed in PBS, cytocentrifuged on poly-L-lysine (PLL)-coated glass slides and then treated with pre-chilled CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES [pH 6.8], 3 mM $MgCl_2$, 1 mM EGTA, 0.5 % Triton X-100, 1.2 mM phenylmethylsulfonyl fluoride and 1.2

mM vanadyl adenosine) for 10 min on ice. Cells were fixed for 15 min at room temperature in PBS containing 4 % paraformaldehyde (PFA) and 15 mM $MgCl_2$. The slides were then washed with PBS and stored in 70 % ethanol at 4 ^{o}C. Cells were re-hydrated in PBS and treated with equilibration solution (50 % formamide, 2 x SSC) for 15 min at room temperature. The hybridization mixture (20~40 ng labeled probe, 6 µg salmon sperm DNA, 3 µg human COT I DNA, 50 % formamide, 10 % dextransulfate and 2 x SSC) was denatured at 75 ^{o}C for 5 min and then 15 µL was applied to each slide. The slide was covered and hybridization performed overnight at 37 ^{o}C. Stringent washing and detection of hybridized DIG-labeled probe were performed as described previously [9, 12]. Cultures were treated for 5 min with a 2.5 mM Bromouridine (BrU; Sigma) pulse and incorporation of BrU into newly-transcribed RNA was detected using mouse anti-BrdU monoclonal antibody (Roche Diagnostics Co., IN) and Alexa 564-conjugated goat anti-mouse IgG antibody (Invitrogen Co., CA).

The epifluorescence images in Fig. 1 were obtained using a Nikon TE2000U microscope equipped with a 100x objective lens (Nikon Plan Fluor, NA 1.30 oil). Images in Figs. 3 and 4 were obtained using a BioRad MRC600 confocal system on a Zeiss Axiovert 135 microscope equipped with a x100 objective (Zeiss, Plan-Neofluor, 1.30 oil). Digital images were visualized in pseudocolors and merged using Adobe Photoshop CS (Adobe Systems Inc., Mountain View, CA).

Results

The commonly-used RNA-FISH procedures remove most of the RNA from the nucleolus.

RNA-FISH can be used to detect specific RNAs *in situ* and most protocols utilize a 70 %

ethanol fixation step to increase probe permeability, as well as performing the hybridization in buffer containing 50 % formamide [10, 11]. Typically, the nucleolus is identified as a faded zone within the propidium iodide (PI)-stained nucleus. However, PI stains both DNA and RNA, and the latter species is enriched in the nucleolus. In fact, cells stained with PI immediately following PFA fixation exhibited brightly stained nucleoli with the nucleoplasm (Fig. 1A, B). Bright staining of the nucleolus did not diminish after the 70 % ethanol treatment (Fig. 1C), but reduced significantly following hybridization (Fig. 1D). Thus, the hybridization procedure removed the majority of RNA from the nucleolus. We then detected newly-transcribed RNA using immunofluorescence of cells pulse-treated with BrU. Following PFA-fixation, we observed signals from both the nucleoplasm and nucleolus (Fig. 1E). However, following treatment with 70 % ethanol, the nucleolar signal diminished and only the nucleoplasmic signal could be observed. Thus, newly-synthesized RNA is removed easily from the nucleolus by 70 % ethanol treatment. These results are summarized in Fig. 2.

Stable association between c-myc RNA and the nucleolus is cell cycle-dependent.

In order to detect the sense strand, we performed RNA-FISH using an antisense RNA probe prepared from the c-*myc* genomic region containing exon 3 (EX3). High levels of c-*myc* RNA were detected in the nucleoli, as well as in many nucleoplasmic foci (for representative images, see Fig. 3B). Since we have found that 70 % ethanol treatment and hybridization remove the majority of RNA species from the nucleolus, detection of a nucleolar signal suggests that c-*myc* RNA forms a stable association with the nucleolus.

Although total intracellular c-*myc* mRNA [13] and protein product [14] levels have been

reported to remain invariant throughout the cell cycle, we detected nucleolar c-*myc* signals in only 73 % of nuclei (77 out of 106) from logarithmically-growing cells. This finding may suggest that nucleolar localization is cell cycle-dependent. Thus, we synchronized the cell cycles of COLO 320DM cells and performed a hybridization using an antisense EX3 RNA probe (Fig. 3). C-*myc* RNA was not detected in mitotic cells, but was present in the nucleoli and nucleoplasmic foci of G1-phase cells. A weak nucleolar c-*myc* RNA signal was detected in a proportion of early S-phase-enriched cells. In contrast, signal was detected only in the nucleoplasm of mid-S-phase-enriched cells and was not present in the nucleoli. These observations suggest that the intranuclear localization of c-*myc* RNA changes during the cell cycle.

Exon- and strand-specific localization of c-myc RNA to the nucleolus.

Next, we prepared strand-specific RNA probes from other portions of human c-*myc* (Fig. 4A) and performed similar analyses to those described above (Fig. 4B - G). Interestingly, the EX1 sense RNA signal was detected exclusively in the nucleoli, whereas signals for sense EX2 and EX3 were found in both nucleoli and nucleoplasmic foci. These results suggest that full-length sense RNA (containing exon 1) accumulates preferentially in the nucleoli, whereas RNA containing only exons 2 and 3 localizes to the nucleoplasmic foci. In addition, we used the EX2 sense probe to detect antisense RNA and observed that it was restricted to the nucleoplasmic foci (Fig. 4E). These results suggest that antisense RNA co-localizes with sense RNA for exon 2, but not exon 1. Since no significant nucleolar signal was obtained with the EX1 sense probe, it is possible that antisense transcription attenuated prior reaching this location. Furthermore, no signal was obtained with the EX3 sense probe, which confirmed the specificity of the

hybridization procedure.

Discussion

Here, we have demonstrated that c-*myc* RNA accumulates in the nucleoli and nucleoplasmic foci. It is likely that the latter accumulation represents a build up of c-*myc* RNA at the inter-chromosomal domain space near the transcription sites (Utani, *et al.*, manuscript in preparation). We found that nucleolar c-*myc* RNA associates tightly with the nucleolus, since a signal remained after most of the RNA was removed from the nucleolus by the hybridization procedure. Interestingly, we observed nucleolar accumulation of RNA containing the non-protein coding exon 1, whereas the RNA lacking this exon remained in nucleoplasmic foci. This finding suggests that exon 1 plays a role in the intranuclear localization of c-*myc* RNA. P2 is the major promoter in undifferentiated, proliferating cells and it drives transcription of a product that contains part of exon 1. In contrast, cells with normal c-*myc* alleles (rearranged or non-rearranged) contain significant amounts of RNA transcribed from the P3 promoter and these do not include exon 1 [15, 16]. Thus, transcripts originating from P2 or P3 may accumulate in the nucleolus or nucleoplasmic foci, respectively. In a previous study, FISH did not detect nuclear localization of a heat-induced transcript from recombinant c-*myc* fused to a heat shock promoter [17]. These results are in agreement with our findings, since we have demonstrated that nucleolar localization is dependent upon exon 1, which was not included in their recombinant construct [18]. We have shown here that the EX2 antisense strand localizes to nucleoplasmic foci, where it may interact with c-*myc* sense strands that lack exon 1. Furthermore, we have shown that the nucleolar accumulation of c-*myc* RNA is regulated with cell cycle progression. c-Myc is a

transcription factor that plays a critical role in growth promotion and our findings strongly suggest that intranuclear localization of c-*myc* RNA is important for post-transcriptional regulation of its expression.

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Figure legends

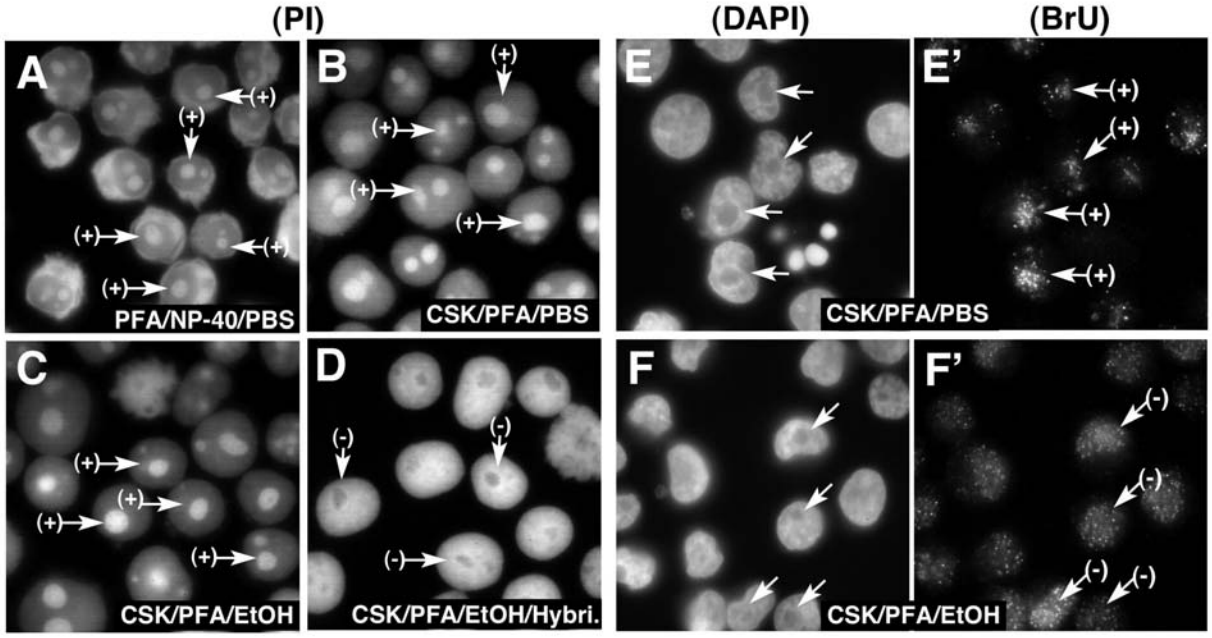
Fig. 1. *RNA-FISH procedures remove RNA from the nucleolus.* Logarithmically-growing COLO 320DM cells (A - D), or cells pulse-treated with BrU for 5 min to label newly-synthesized RNA (E, F), were harvested and cytocentrifuged on glass slides. (A) Cells were fixed with PFA, treated with 0.5 % NP-40 in PBS and stored in PBS. (B - F) Cells were treated with CSK buffer and fixed with PFA using the RNA-FISH protocol. These slides were stored in PBS (B and E) or immersed in 70 % ethanol at room temperature for 30 min (C, D and F). One slide (D) was subjected to

hybridization, according to the RNA-FISH protocol. (A - D) DNA and RNA were stained with PI (red fluorescence). (E and F) DAPI staining of DNA and BrU incorporation were detected by blue and green fluorescence, respectively. Arrows show nucleoli and (+) or (-) indicates the presence or absence of a fluorescent signal.

Fig. 2. Schematic showing that hybridization and ethanol treatment remove most of the RNA or newly-synthesized RNA from the nucleolus, respectively.

Fig. 3. Cell cycle dependent changes in c-*myc* RNA localization. COLO 320DM cells arrested at the prometaphase (A) were released into G1 phase and harvested after 4 h (B). Cells arrested at the G1/S-boundary were released synchronously into S phase and harvested after 1 h (C) or 6 h (D), representing early- or middle-S phase, respectively. Harvested cells were fixed and hybridized with a c-*myc* antisense EX3 RNA probe. The aneuploidy of this cell line causes different cell sizes at the same phase in the cell cycle. DNA and RNA were counterstained with PI (red). Arrows show nucleoli and (+) or (-) indicates the presence or absence of a c-*myc* RNA signal. Bars = 10 μm.

Fig. 4. Localization of different c-*myc* RNA species at specific nuclear locations. (A) The genomic structure of c-*myc*. Open and closed boxes represent the non-coding and protein-coding exons, respectively. Sense (P0-P3) and antisense (AP) promoters are indicated with arrows showing the direction of transcription. The regions identified by red lines (EX1 ~EX3) were cloned into plasmid vectors, from which antisense and sense RNA probes were prepared. These probes were hybridized to logarithmically-growing COLO 320DM cells. Representative images are shown in B to G. Bars = 10 μm.


(PI)
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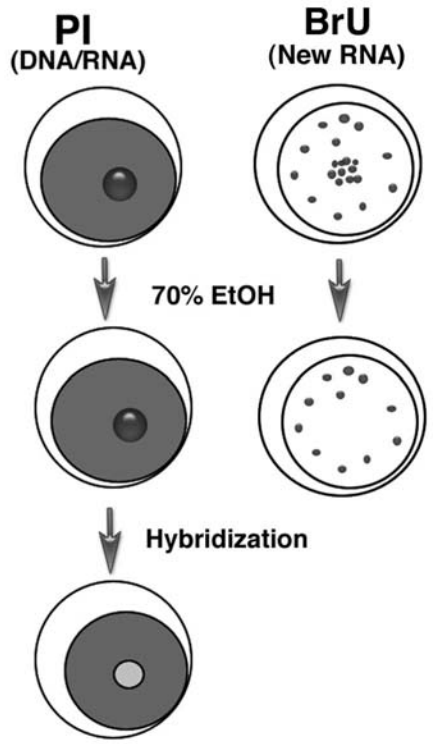
CSK/PFA/EtOH/Hybri.
CSK/PFA/EtOH

Fig. 1.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BrU
(New RNA)
70% EtOH
Hybridization

Fig. 2.

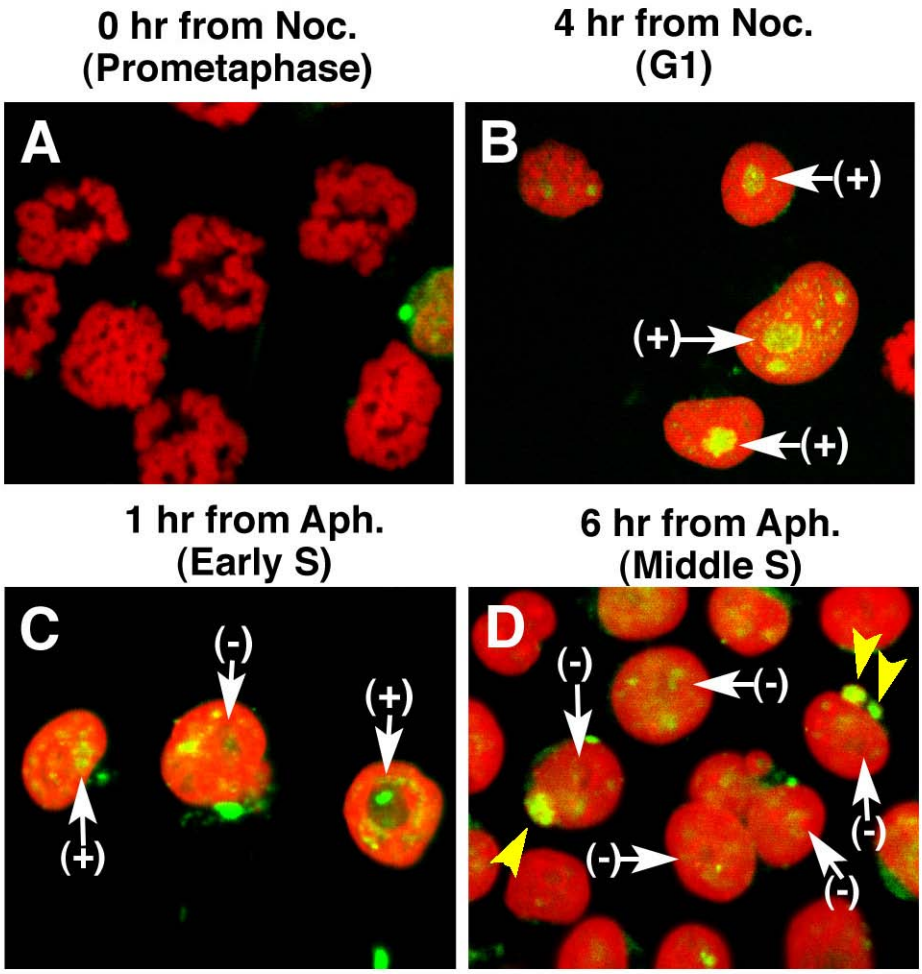
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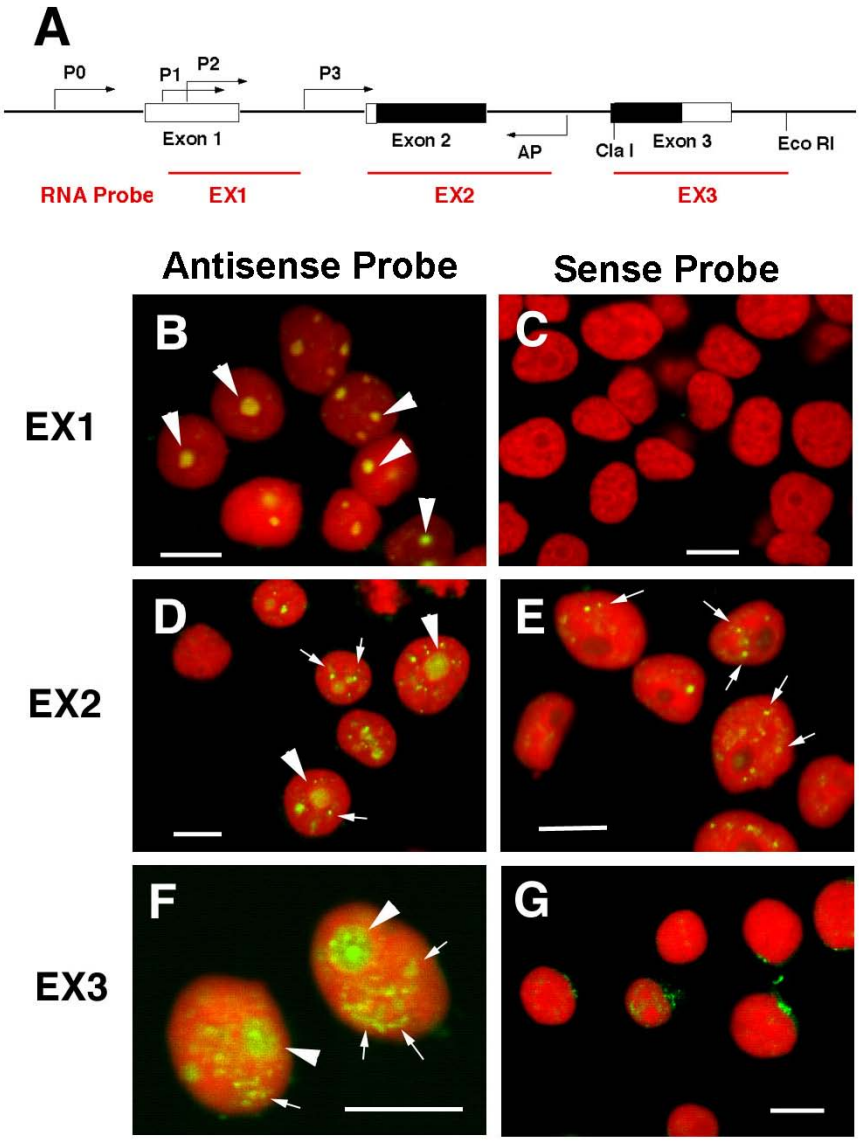
Fig. 3.


A
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Exon 3
Eco RI
RNA Probe
EX1
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EX3
Antisense Probe
Sense Probe
B
C
D
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G
EX1
EX2
EX3

Fig. 4.