A cell synchronization protocol was established in which global and individual mRNA translational efficiency could be examined. While global translational efficiency was reduced in mitotic cells, ~3% of mRNAs remained predominantly associated with large polysomes during mitosis, as determined by cdNA microarray analyses. The 5' non-coding regions of six mRNAs were shown to contain internal ribosome entry sites (IRES). However, not all known mRNAs that contain IRES elements were actively translated during mitosis, arguing that specific IRES sequences are differentially regulated during mitosis.

Cells of higher organisms can regulate gene expression at the translational level in response to a wide variety of stimuli and conditions. While much is known about the control of translational initiation and elongation in response to nutritional deprivation (1, 2) and environmental stress (2), the role of translational control in mammalian cell cycle progression is not well understood. It has long been known that specific proteins are needed at specific times during the cell cycle to ensure cell cycle progression. For the most part, the expression of these cell cycle-specific proteins is thought to be regulated at the transcriptional or posttranslational level (3).

In cultured mammalian cells arrested at G2 and M phases, the rate of total protein synthesis was markedly decreased to about 25% of the rate in non-arrested, cycling cells (4). This reduction was shown to be, at least partly, due to inhibition of the initiation step of polypeptide synthesis (4–6). Subsequently, several eukaryotic initiation factors that regulate the assembly of 40 S ribosomes at the 5' ends of capped mRNAs were observed to change their phosphorylation status (7–9). It has been shown that specific IRES sequences are differentially regulated during mitosis.

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The abbreviations used are: IRES, internal ribosome entry site; NCR, non-coding region; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; RACE, rapid amplification of cdNA ends; PARP, poly(ADP-ribose) polymerase; ODC, ornithine decarboxylase.

‡‡ The nucleotide sequences of all inserted fragments were obtained. The 5' ends of capped mRNAs (4, 11–14) or mRNAs which lack significant structures in their 5' non-coding regions (5'NCRs), such as the mRNAs containing the late leader of adenovirus (15), were found to be selectively translated during mitosis due to their lessened requirement for eIF4F. More recently, additional IRES-containing mRNAs, those encoding ornithine decarboxylase and kinase p58PTSLRE, have been reported to be selectively translated during G2/M of the cell cycle in cultured cells (11, 12). These findings raise the question whether all IRES-containing genes are preferentially translated during mitosis and whether any of these encoded products play roles in cell cycle progression. We have begun to address these questions by genomic analysis of cellular mRNAs that are associated with mitotic polysomes and are, thus, predicted to be translated during the overall translation repression in mitosis. We determined that many, but not all, IRES elements are present in mRNAs which are selectively translated during mitosis.

EXPERIMENTAL PROCEDURES

Cell Synchronization and Transfection Protocols—HeLa cells were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen). To synchronize cells at the G1/S boundary, cells were treated at 50% confluence with 2 mM thymidine (Sigma) or 5 μg/ml aphidicolin (Sigma). After 16 h, cells were washed and fed with fresh medium for 6 h before the addition of either 0.6 μg/ml colcemid (Invitrogen) or nocodazole (Sigma; Ref. 16). Synchronization was monitored by flow cytometry analysis after DNA staining with propidium iodide. To measure protein synthesis rates in nonsynchronized and mitotic arrested cells, cells were incubated in DMEM with 100 μCi/ml [35S]methionine/cysteine for 10 min. Extracts were prepared and processed as described previously (17).

DNA plasmids were transfected into 50–60% confluent cells with the Lipofectin reagent (Invitrogen). After a 24-h incubation, luciferase assays were performed or RNA samples for Northern analyses were prepared.

Indirect Immunofluorescence Analysis—Cells were grown on 12-mm coverslips, fixed for 5 min in 100% methanol at −20 °C, and permeabilized in phosphate-buffered saline with 0.2% Triton X-100. Following fixation and permeabilization, cells were washed with PBS and incubated in PBS containing 3% bovine serum albumin, 0.1% Triton X-100, and 5% calf serum for 40 min at 25 °C. Coverslips were then incubated with diluted rat antibodies against α-tubulin (a gift from P. Jackson, Stanford University) in IF buffer (PBS with 3% bovine serum albumin and 0.1% Triton X-100) for 30 min at 25 °C. Coverslips were then incubated with diluted rat antibodies against α-tubulin (a gift from P. Jackson, Stanford University) in IF buffer (PBS with 3% bovine serum albumin and 0.1% Triton X-100) for 30 min at 25 °C. After washing, coverslips were inverted into the IF buffer with Texas Red-conjugated donkey anti-rat antibody for 30 min at 25 °C. Cover slips were washed twice with PBS, and once with PBS containing 0.2 μg/ml Hoechst 33258, drained, and mounted in vectashield mounting medium (Vector Laboratories) onto glass slides. Cells were viewed using an Olympus BX-60 fluorescent microscope. Pictures were acquired using a Hamamatsu Orca digital camera and Image Pro plus software with 40× or 60× objectives.

5' RACE and Dicistronic Plasmid Constructions—The 5' NCRs of selected mRNA species were converted to cdNA copies using the First-Choice RLM-RACE Kit (Ambion). The cdNA fragments were amplified by the Advantage-GC PCR kit (Clontech) and directly cloned into pcRR2.1-topo vector (Invitrogen). The nucleotide sequences of all inserted fragments were obtained. The 5' NCRs were amplified by the PCR with 5' primer containing EcoRI sequences and 3' primers con-
Fig. 1. Translation reduction during mitosis in HeLa cells. A, flow cytometry analysis of HeLa cells that were arrested by the sequential treatments of thymidine and colcemid or thymidine and nocodazole. x axis, Cy5PE propidium iodide; y axis, cell numbers. B, non-synchronized cells and thymidine/colcemid-arrested cells. Left, ×100 image of non-synchronized and thymidine/colcemid-arrested HeLa cells. Right, non-synchronized cells and thymidine/colcemid-arrested cells stained with antibodies against α-tubulin and Hoechst; ×100 fluorescence image. C, protein synthesis reduction in mitotic arrested cells. [35S]Methionine/cysteine incorporation into non-synchronized and mitotic cells, arrested with either thymidine/colcemid (T/C) or thymidine/nocodazole (T/N), was determined by trichloroacetic acid precipitation. The percentage of the radiolabel incorporation compared with of non-synchronized cells is shown. D, Western blot analysis of PARP protein in non-synchronized and mitotic-arrested cells. Cells were arrested at mitosis by treatment with aphidicolin/colcemid (A/C), thymidine/colcemid (T/C), or thymidine/nocodazole (T/N) in the presence or absence of apoptosis inhibitor benzylxycarbonyl-VAD-fluoromethylketone. Non-synchronized HeLa cells were used as control. The apoptosis inhibitor benzylxycarbonyl-VAD-fluoromethylketone was added to the culture 30 min before harvest at a concentration of 50 μM. The arrow refers to the intact PARP proteins.
taining NcoI sequences. These fragments were inserted into dicistronic SV40/T7 plasmid, c-53, between EcoRI and NcoI sites, or a T7 promoter-containing dicistronic vector, c-84, as described previously (18).

Northern Blot Analyses—Total RNA was isolated using the TRIzol Reagent (Invitrogen) and analyzed as described previously (17). Radiolabeled DNA hybridization probes were generated using the RadPrime Kit (Invitrogen).

Polyosomal Analysis—Polyosomal mRNA was prepared as described previously (17). Briefly, cells were incubated with 0.1 mg/ml cycloheximide for 3 min at 37 °C before being harvested. Non-synchronized control cells were washed with PBS, harvested directly on the plate in lysis buffer (15 mm Tris-HCl, pH 7.4, 15 mm MgCl₂, 0.3 µg/ml NaCl, 1% Triton X-100, 0.1 mg/ml cycloheximide, 1 mg/ml heparin), and transferred to Eppendorf tubes. The mitotic cells were shaken off from plates and pelleted at 1000 rpm for 3 min. Mitotic cells were washed in PBS and lysed in lysis buffer for 10 min on ice. The nuclei and debris were separated by sucrose gradient centrifugation. The fractions were collected from the top using an ISCO fraction collector. RNAs were collected in 4 M guanidine HCl, precipitated after volumes were collected from the top using an ISCO fraction collector and lysed in lysis buffer for 10 min on ice. The supernatants were loaded onto 10–30% sucrose gradients composed of same extraction buffer lacking Triton X-100. The gradients were sedimented at 35,000 rpm for 160 min in a SW41 rotor at 4 °C. Fractions of equal volumes were collected from the top using an ISCO fraction collector system.

Western Blot and Immunoprecipitation Analyses—Proteins were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Poly(ADP-ribose) polymerase (PARP) was detected with anti-PARP antibody number SC7150 (Santa Cruz Biotechnology) as described previously (19). To examine newly synthesized proteins, cells were grown in DMEM lacking methionine and cysteine (Invitrogen) with previously (17). Briefly, cells were incubated with 0.1 mg/ml cycloheximide for 3 min at 37 °C before being harvested. Non-synchronized control cells were washed with PBS, harvested directly on the plate in lysis buffer (15 mm Tris-HCl, pH 7.4, 15 mm MgCl₂, 0.3 µg/ml NaCl, 1% Triton X-100, 0.1 mg/ml cycloheximide, 1 mg/ml heparin), and transferred to Eppendorf tubes. The mitotic cells were shaken off from plates and pelleted at 1000 rpm for 3 min. Mitotic cells were washed in PBS and lysed in lysis buffer for 10 min on ice. The nuclei and debris were removed by centrifugation at 12,000 × g for 10 min, and the supernatants were loaded onto 10–50% sucrose gradients composed of same extraction buffer lacking Triton X-100. The gradients were sedimented at 35,000 rpm for 160 min in a SW41 rotor at 4 °C. Fractions of equal volumes were collected from the top using an ISCO fraction collector system.

RESULTS
Reduction of Protein Synthesis in G2/M-arrested HeLa Cells—To investigate translational control during mitosis, HeLa cells were synchronized in mitosis by a sequential block with thymidine and colcemid or with thymidine and nocodazole. Mitotic cells were shaken off from the plates, and the efficiency of synchronization was monitored by flow cytometry after propidium iodide staining. About 80–90% of cells treated with thymidine-colcemid or thymidine-nocodazole displayed a double content of DNA, confirming their arrest (Fig. 1A). To identify the specific mitotic phase in which cells were arrested, the microtubule structure of thymidine/colcemid-arrested cells and non-synchronized cells was visualized by immunofluorescence staining using antibodies directed against α-tubulin (Fig. 1B). Loss of functional spindle structures and condensed chromosomes in treated cells revealed that these mitotic cells were arrested at prophase or metaphase of mitosis.

To compare the translational efficiencies in mitotically arrested and non-synchronized cells, cells were pulse-labeled with [35S]methionine/cysteine, and the incorporation of radiolabeled amino acids was measured. As reported previously (4), both thymidine/colcemid- and thymidine/nocodazole-arrested cells showed a reduced incorporation of radiolabeled amino acids compared with non-synchronized cells (Fig. 1C).

Fig. 2. Association of mRNAs with polysomes in non-synchronized and mitotically arrested HeLa cells. Lysates were fractionated by sucrose gradient centrifugation. The fractions from the top to the bottom of the sucrose gradient are displayed from left to right. The absorbance profile at 260 nm is shown, and the arrow denotes the migration of 40 and 60 S ribosomal subunits. The distributions of β-actin and transforming growth factor-β mRNA in each fraction, obtained after Northern analysis, are shown. The Northern analyses were performed three times.

Fig. 3. Representative histogram displaying the polysomal distribution of mRNA species (x) in mitotic cells compared with that in non-synchronized cells. The microarray score index S(x) was calculated as follows. Non-synchronized: I(x) = LOG2 (pol(x)/pol(x)); mitosis: M(x) = LOG2 (pol(x)/pol(x);) (where i indicates an individual array measurement using RNA extracted from non-synchronized cells (i = 1, 2, 3), j indicates an individual array measurement using RNA extracted from mitosis cells (j = 1, 2, 3), x indicates an individual species with a specific IMAGE ID on each array, S(x) = M(x) − I(x); σ is the standard deviation. The arrows point to the positions of the mean S(x) and 2σ.

Microarray score

mean=0.80; standard deviation=0.91

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Fig. 4. Polysomal distribution of selective mRNAs in non-synchronized and mitotic arrested cells. A, polysomal distributions of β-actin, GRP58, NAP1L1, NPM1, La, and ODC mRNAs were displayed after Northern hybridization as described in the legend to Fig. 2. Note that mRNAs were visualized after reprobing of the blot shown in Fig. 2. Thus, data showing distribution of β-actin are identical in Figs. 2 and 4. B, quantification of mRNA distributions of β-actin, ODC, NAP1L1, and GRP58. The data points represent the percent intensity of each fraction relative to the total combined intensity of all fractions for each gradient. The Northern analyses were performed three times.
It has been recently cloned that many synchronization protocols can induce apoptosis (20, 21). To examine whether the synchronization protocols used here triggered cells to enter apoptosis, caspase-induced cleavage of PARP was measured. Fig. 1D shows that little cleavage of PARP occurred in either thymidine/colcemid- or thymidine/nocondazole-treated cells. In contrast, notable amount of PARP was cleaved in another synchronization protocol, i.e. treatment of cells with aphidicolin and colcemid. These findings suggest that the global reduction of protein biosynthesis that was observed in thymidine/colcemid- and thymidine/noconazole-treated cells was not a result of ongoing apoptosis.

Reduced Association of Cellular mRNAs with Mitotic Polyribosomes—To examine the translation status of both total and individual mRNAs, cytoplasmic extracts from interphasic- and mitotic-arrested cells were fractionated in sucrose gradients (17). Fig. 2 shows that, in non-synchronized cells, most ribosomes were associated with mRNAs, sedimenting with polyribosomal fractions 8–10. In contrast, most ribosomes in mitotic extracts had dissociated from mRNAs, sedimenting as free 40 and 60 S subunits (Fig. 2). This finding argues for mitotic inhibition at the initiation step of translation.

To monitor the association of individual mRNAs with ribosomes, the distribution of individual mRNAs in the polysome gradient was visualized by Northern blot analysis using gene-specific hybridization probes. mRNAs encoding β-actin and transforming growth factor-β were mostly associated with fractions 10 and 9, respectively, in non-synchronized cells. In contrast, these mRNAs shifted to the lower molecular weight fractions in mitotically arrested cells, indicating that these mRNAs were less efficiently translated in mitosis than in interphase.

Identification of Mammalian mRNAs Species That Are Preferentially Associated with Mitotic Polyribosomes—To identify individual mRNA species which are selectively translated during mitosis, the polysomal distribution of mRNAs was examined using human cDNA microarray analyses (22, 23). Specifically, the amount of individual cDNAs obtained from RNA in fractions 4–7 was compared with that obtained from RNA in polynomials of 9 and 10 (Fig. 2). Statistical analyses indicated (Fig. 3) that the majority of mRNAs shifted toward the lower molecular weight polysomal fractions during mitosis, arguing that the mitotic sedimentation profiles of β-actin and transforming growth factor-β mRNAs (Fig. 2) reflected an overall diminished association with ribosomes of mRNAs. However, at least 3% of the mRNA species (49 genes out of 1494 genes) continued to be associated with similar or more numbers of ribosomes in mitotic extracts, determined both by statistical analysis (Fig. 3) and Northern blot analysis (Fig. 4). Selected positive candidate genes are listed in Table I. Approximately one-third of these genes encode nuclear proteins, many of which are predicted to play roles in RNA metabolism (Table I).

We further substantiated the polysomal distribution of some of the mRNAs identified in the microarray analyses by Northern blot analysis using gene-specific hybridization probes. In contrast to β-actin mRNAs, mRNAs encoding the glucose regulated 58-kDa protein (GRP58), nucleosome assembly protein 1-like 1 (NAP1L1), nucleophosmin (NPM1), and the autoantigen La sedimented with the same polysomal fractions in non-synchronized and arrested cells (Fig. 4, A and B). In contrast to the findings reported by Pyronnet et al. (12), ornithine decarboxylase (ODC) mRNA shifted, like β-actin mRNA, to lower molecular weight polysomal fractions during mitosis (Fig. 4, A and B). Specifically, the majority ODC mRNAs was associated with ~4–5 ribosomes (fraction 9) in non-synchronized cells. However, the bulk of ODC mRNA associated with only 1–3 ribosomes (fractions 7 and 8) in mitotically arrested cells. While the reason for this discrepancy is not known, different cell lines or cell synchronization protocols may have accounted for the observed difference.

It has been recently found that many synchronization protocols can induce apoptosis (20, 21). To examine whether the synchronization protocols used here triggered cells to enter apoptosis, caspase-induced cleavage of PARP was measured. Fig. 1D shows that little cleavage of PARP occurred in either thymidine/colcemid- or thymidine/nocondazole-treated cells. In contrast, notable amount of PARP was cleaved in another synchronization protocol, i.e. treatment of cells with aphidicolin and colcemid. These findings suggest that the global reduction of protein biosynthesis that was observed in thymidine/colcemid- and thymidine/nocondazole-treated cells was not a result of ongoing apoptosis.

### Table I

| Gene name and Probes | IMAGE ID |
|----------------------|----------|
| Multiple IMAGE clones of a same gene on the DNA microarrays were identified as polysomal-associated candidates during mitosis. | |
| | |
| **Selective candidates of polysomal-associated mRNAs during mitosis identified by cDNA microarrays** | |
| | |
| **Gene name** | **IMAGE ID** |
| Eukaryotic translation initiation factor 4 γ, 1 | 25,988 |
| Eukaryotic translation initiation factor 3, subunit 10 (e, 150/170 kDa) | 147,279 |
| Eukaryotic translation initiation factor 4B | 147,275 |
| Membrane and secreted proteins | |
| Tumor rejection antigen (gp96) | 26,519 |
| Glucose regulated protein, 58 kDa (GRP58) | 135,083 |
| Calnexin | 268,178 |
| Immediate early response 3 | 810,724 |
| Nuclear proteins | |
| RAN-binding protein 7 | 78,695 |
| Parathymosin | 81,087/20,729/890,603* |
| Polymerase I and transcript release factor | 809,472 |
| Nuclear RNA-binding proteins | |
| Nucleophosmin (nucleolar phosphoprotein B23, numatrin) (NPM1) | 884,301 |
| Nucleosome assembly protein 1-like 1 (NAP1L1) | 275,871 |
| Splicing factor, arginine/serine-rich, 46-kDa (SRF46) | 416,951 |
| DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5 (RNA helicase, 68 kDa) | 126,775 |
| PAI-1 mRNA-binding protein | 268,178/236,055* |
| Sjogren syndrome antigen B (autoantigen La) (La) | 49,970 |
| Heterogeneous nuclear ribonucleoprotein A/B (hnRNP/A/B) | 345,833 |
| Structure proteins | |
| Vimentin | 840,511 |
| Signaling proteins | |
| ras homolog gene family, member B | 277,305 |
| Cysteine-rich, angiogenic inducer 61 (Cyr61) | 388,488 |
| Misc/others | |
| Amyloid β (A4) precursor protein (protease nexin-II, Alzheimers disease) (ABETA) | 526,616/856,575* |
| Heat shock 70-kDa protein 8 | 884,719/209,383/951,287* |
| Heat shock 70-kDa protein 4 | 856,567 |

* Multiple IMAGE clones of a same gene on the DNA microarrays were identified as polysomal-associated candidates during mitosis.
sized protein compared with total translational efficiency during mitosis. The amounts of total nucleophosmin, vimentin, and La mRNAs were very similar in non-synchronized and mitotic extracts. While the translational efficiencies of nucleophosmin and La mRNAs were somewhat enhanced during mitosis, the mitotic translational efficiency of vimentin mRNA was more pronounced (Fig. 5, A and B) compared with the bulk of cellular mRNAs. These findings argue that the polysomal association of these mRNAs reflected translation by ribosomes.

**mRNAs Preferentially Translated during Mitosis Contain IRES Elements**—The cDNA microarray analyses identified two genes known to contain IRES elements that were preferentially translated during mitosis. Specifically, La and Cyr61 mRNAs were previously identified to be associated with polysomes during poliovirus infection (23). To examine whether the 5′-NCRs of identified mitotic polysomal-associated mRNAs could mediate cap-independent translation via an IRES-mediated mechanism, cDNA copies of the 5′-NCRs of several identified candidate mRNAs were obtained by 5′ RACE. As expected (10), there were no strong primary consensus sequences among them by multiple or pairwise alignment analyses. Also, the 5′-NCRs were of the same length and similar GC nucleotide content than the average mammalian mRNA. For comparison, cDNA copies were obtained from six mRNAs that represented the majority of cellular mRNAs that were not associated with polysomes during mitosis (β-actin, PMSA1, CSTB, HNRPA1, NUBS, and RPS5).

The cDNAs encoding the various 5′-NCRs were inserted between two cistrons in dicistronic plasmids, which can direct the expression of dicistronic mRNAs that contain the coding regions for Renilla and firefly luciferase as the first and second cistrons, respectively (Fig. 6A) (23). The first, Renilla luciferase, cistron is predicted to be translated by a cap-dependent initiation mechanism, while the second, firefly luciferase, cistron should be translated if preceded by an IRES element. The various dicistronic plasmids were transfected into HeLa cells and luciferase activities were measured. The six 5′-NCRs of mRNAs that were associated with fewer ribosomes in mitosis than in non-synchronized cells expressed the second cistron firefly luciferase either similarly or in 2–3-fold higher amounts as the dicistronic control mRNA construct that lacked an intergenic inserted region (Fig. 6B). Therefore, these 5′-NCRs did not contain significant IRES activities. In contrast, six out of seven 5′-NCRs from preferentially mitotic polysome-associated mRNAs significantly promoted the expression of firefly luciferase encoded in the second cistron, yielding 7–30-fold higher rates than seen in cells transfected with dicistronic actin 5′-NCR-containing mRNAs (Fig. 6B). To examine whether expression of the second cistron resulted from functionally monocistronic transcripts generated by nuclease activity, cryptic splicing, or expression of an unknown promoter in the insert 5′-NCR-encoding cDNA, the size and integrity of expressed dicistronic mRNAs from transfected cells were inspected by

**TABLE II**

| Summary of 5′-NCRs |
|-------------------|
| None of these 5′-non-coding regions contains AUG codons. |

| mRNA species that remain associated with a similar number of polysomes during mitosis | Gene symbol | 5′-NCR length | GC content % |
|---|---|---|---|
| Sjogren syndrome autoantigen B, La | La | 105 | 62.8 |
| Cysteine-rich, angiogenic inducer 61 | Cyr 61 | 223 | 78.0 |
| Nucleophosmin | NPM1 | 97 | 59.8 |
| Nucleosome assembly protein 1-like 1 | NAP1L1 | 139 | 63.4 |
| Amyloid β A4 precursor protein (ABETA) | ABETA | 147 | 76.0 |
| Glucose-regulated protein, 58 kDa | GRP58 | 113 | 75.9 |
| Heterogeneous nuclear ribonucleoprotein A/B | hnRNP A/B | 223 | 73.9 |
| Vimentin | Vimentin | 146 | 76.7 |

| mRNA species that are associated with fewer ribosomes during mitosis | Gene symbol | 5′-NCR length | GC content % |
|---|---|---|---|
| β-Actin | ACT | 87 | 74.7 |
| Proteasome (prosome, macropain) subunit, alpha1 (NM 002786) | PMAS1 | 103 | 58.5 |
| Cystatin B (NM-00010) | CSTB | 109 | 73.4 |
| Heterogeneous nuclear ribonucleoprotein A1 (X12671) | HNRPA1 | 104 | 57.9 |
| NADH-ubiquinone dehydrogenase 1 β subcomplex (AF2610900) | NDUFB | 84 | 72.4 |
| Ribosomal protein S9 (NM 001009) | RPS9 | 53 | 64.3 |
Mitotic IRES Elements

Northern blot analysis. Fig. 6C shows that each plasmid directed the synthesis of predominantly full-length mRNAs, arguing that full-length dicistronic mRNAs were the major templates for the second cistron translation. To test this hypothesis more rigorously, the SV40 promoters were deleted in all plasmids and luciferase activities were measured after transfection of these promoterless plasmids into cells. All but one of the constructs expressed little to no luciferase activity (data not shown). Surprisingly, the NAP1L1 leader cDNA sequences could mediate second cistron translation at the level of 10-fold higher than background (data not shown), demonstrating that smaller mRNAs may have contributed to the translation of the second firefly luciferase cistron in this case. This finding exemplifies that by Northern analysis an undetectable amount of functional monocistronic RNA can be generated in the dicistronic assay system, and additional control experiments are warranted to claim IRES activity. To test whether the 5’-NCR of NAP1L1 mRNA carried a bona fide IRES activity, full-length dicistronic control, and NAP1L1- or NPM1-NCR-containing mRNAs were generated in vitro by T7 RNA polymerase, and mRNAs were translated in HeLa S10 translation extracts. Fig. 6D shows that relative expression level of the second cistron in dicistronic NAP1L1-containing mRNA was more than 100-fold higher than in mRNAs lacking intercistronic sequences. This finding suggests that the NAP1L1 leader does contain IRES activity that is significantly higher than the various control 5’-NCRs (Fig. 6B). Overall, these findings indicate that IRES activities can be active during mitosis when translation of the bulk of the cellular mRNAs has declined.

DISCUSSION

We have studied translational control in mitotically arrested HeLa cells using cell synchronization protocols that minimized the accumulation of apoptotic cells. Specifically, we examined whether selected individual mRNAs could be preferentially translated during the global translational repression during mitosis. To this end, polysomal mRNAs were analyzed by cDNA microarray analysis. The ribosome density on a particular mRNA was used as the indicator of the relative translational efficiency of the mRNA. By separating the mRNAs into non-polysomal fractions and polysomal fractions and comparing their relative abundance by microarray analysis, we examined the translation state of individual mRNAs in non-synchronized cells and mitotically arrested cells. The 5’-NCRs of selected mitotic polysome-associated mRNAs were 100–200 nucleotides in length (Table II). Six out of seven of those selected leader sequences functioned as IRES elements in dicistronic assays, suggesting that internal initiation is more efficient than cap-dependent initiation during mitosis. One possibility to this selective translational control is that cap-dependent and internal translation compete with each other for components of the translation apparatus. Many of the mRNAs which were associated with mitotic polysomes encoded nuclear RNA binding proteins, such as the La autoantigen or hnRNP.

Because certain nuclear RNA-binding proteins have been identified to stimulate IRES activity, such as La and hnRNP C (24–26), selective up-regulation of IRES-containing mRNAs could be favored by the increased accessibility of these nuclear factors during mitosis. The target IRES-containing mRNAs may encode proteins that are important to aid in cell cycle progression and facilitate specific IRES-mediated translation during mitosis in an autoregulatory manner.

However, not all of the known cellular IRES-containing messages were associated with mitotic polysomes. For example, whereas IRES-containing La (18) and Cyr61 (23) mRNAs were preferentially detected in polysomal fractions during mitosis, IRES-containing c-Myc (27, 28) (not shown) and ODC (12) mRNAs were not. These findings seem to be, at first glance, at odds with observations reported by Pyronnet and Sonenberg (8) who used a sequential thymidine and aphidicolin block to synchronize HeLa cells at S phase, following cell cycle progression through the G2/M phase after release of the drugs. Synthesis of
ODC protein peaked 8 h after release from S phase, but dropped dramatically thereafter (12). It is possible that the up-regulation of ODC mRNA and protein occurred in G2, before cells entered mitosis. Because we focused in the present study on the translation activity of mRNAs in cells arrested in mitosis, up-regulation of ODC in G2 would have not been scored in our assays. Nevertheless, it is clear from all studies (8, 11) that IRES element can be regulated in a cell cycle-specific manner and that IRESs are preferentially used by the mitotic translation apparatus. The task will now be to identify the different regulatory elements in mitotically active IRES elements and the proteins and cellular structures that modulate their activity. The outcome of these lines of investigation will determine roles for the internal initiation mechanism in cell cycle progression and point to posttranscriptional circuits that regulate cell growth.

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