6148–6156 *Nucleic Acids Research, 2003, Vol. 31, No. 21*
DOI: 10.1093/nar/gkg794

A role for c-Myc in the regulation of ribosomal RNA processing

Isabel Schlosser, Michael Hölzel, Marlies Mürnseer, Helmut Burtscher[1], Ulrich H. Weidle[1] and Dirk Eick*

GSF National Research Centre for Environment and Health, Institute of Clinical Molecular Biology and Tumor Genetics, MarchioninistraÃe 25, 81377 Munich, Germany and [1]Roche Diagnostics GmbH, Department TR-ON, 82372 Penzberg, Germany

Received July 25, 2003; Revised and Accepted August 28, 2003

ABSTRACT

The proto-oncogene c-*myc* encodes a basic helix–loop–helix leucine zipper transcription factor (c-Myc) that has a profound role in growth control and cell cycle progression. Previous microarray studies identified various classes of c-Myc target genes, including genes involved in ribosome biogenesis. By screening the human B-cell line P493-6 and rat fibroblasts conditionally expressing c-Myc, we could substantially extend the list of c-Myc target genes, particularly those required for ribosome biogenesis. The identification of 38 new c-Myc target genes with nucleolar function, prompted us to investigate processing of ribosomal RNA (rRNA). Using pulse–chase labelling experiments we show that c-Myc regulates the efficiency of rRNA maturation. In serum-stimulated P493-6 cells, only the processing of the 47S rRNA precursor to mature 18S and 28S rRNA, but not the synthesis of the 47S transcript, was dependent on the presence of c-Myc. As processing of rRNA is sensitive to inhibition of cyclin-dependent kinase (cdk) activity by roscovitine, we conclude that c-Myc regulates cell growth and proliferation by the coordinated induction of cdk activity and rRNA processing.

INTRODUCTION

The proto-oncoprotein c-Myc dimerizes with the helix–loop–helix leucine zipper protein Max. c-Myc/Max heterodimers bind DNA at the E-box sequence motif CACGTG and related sequences, thereby modulating the transcriptional activity of genes (1–3). c-Myc plays a key role in the regulation of cell proliferation, growth, differentiation and apoptosis (3).

The role of c-Myc in growth control varies depending on the cellular context. In a murine model, constitutive expression of a c-*myc* transgene under control of the Ig heavy-chain enhancer increases the cell size of B-lymphocytes at all stages of B-cell development, accompanied by an increase in protein synthesis (4). Similarly, activation of a conditional c-*myc* in the human B-cell line P493-6 induces cell growth, even if cell cycle entry is blocked by the cyclin-dependent kinase (cdk) inhibitor roscovitine (5). Expression of ectopic c-*myc* in primary mouse liver cells is accompanied by enlarged and dysmorphic hepatocytes in the absence of significant cell proliferation (6). These results suggest that c-Myc can govern a cellular growth programme uncoupled from proliferation. However, conditional knockout of c-*myc* in mice only prevented cell cycle entry but not growth of activated T cells (7). In *Drosophila*, reduced levels of dMyc result in hypotrophy (reduced cell size) (8), whereas in mice, reduction of c-Myc levels results in hypoplasia (decrease in cell number) (7).

Numerous reports have been published in recent years that have identified various classes of c-Myc target genes, including genes involved in ribosome biogenesis (3,9). The synthesis of ribosomes is one of the major cellular activities that takes place primarily in the nucleolus. There the rRNA genes are transcribed as precursors, which undergo processing and covalent modification. These processes involve many cellular components acting either alone or as part of a complex. Some components are directly involved in the modification and cleavage of the precursor rRNA, while others direct the packaging of the rRNA into ribosome subunits (10). The rate of ribosome formation is precisely regulated and coordinated with the physiological and developmental changes of a cell (11). Experimental evidence suggests that the basic outline of rRNA processing and ribosome biogenesis are conserved throughout eukaryotes (12). Notably, increased synthesis of ribosomes is often due to the more efficient processing of the 47S precursor rRNA rather than to the increased synthesis of the precursor (13). Here we show that c-Myc regulates the efficiency of rRNA processing.

MATERIALS AND METHODS

Cell culture

Establishment and cell culture of P493-6 cells carrying p*myc*-tet (tet-off system) are described elsewhere (5). To arrest the cells, 0.1 µg/ml tetracycline (tet) was added to medium containing 0.25% FCS for 72 h. For stimulation, cells were washed three times with PBS containing 10 or 0.25% FCS with or without tetracycline. Establishment and cell culture of the

*To whom correspondence should be addressed. Tel: +49 89 7099512; Fax: +49 89 7099500; Email: eick@gsf.de

Table 1. List of induced target genes with nucleolar localisation

Accession number P493-6:		Name	Yeast	Function	Signal ratio
X56597	a,b	fibrillarin	Nop1	Component of snRNP, associated with U3, U8, U13 snoRNAs	1.8
M60858	a,b	nucleolin	Nsr1p	Ribosome biogenesis	2.2
AA191576	a,b	nucleophosmin B23		Ribosomal protein assembly	2.4
AI144254	b	EST	Rrp4	3′→5′ exoribonuclease, subunit of the exosome complex	2.1
AL039469	b	exosome component Rrp41	Rrp41	3′→5′ exoribonuclease, subunit of the exosome complex	2.1
AI971527	b	exosome component Rrp46	Rrp46	3′→5′ exoribonuclease, subunit of the exosome complex	5.5
U41387	a,b	nucleolar protein GU alpha		RNA helicase II/Gu (DDX21), a DEAD-box enzyme	4.4
AI623176	b	nucleolar protein GU beta		Paralogue of GU alpha	1.4
AI742046	b	nucleolar phosphoprotein	Nopp34	Interacts with Ki-67 antigen (MKI67)	2.4
U86602	a,b	nucleolar protein p40	Ebp2	Ribosomal RNA processing in yeast, EBNA1 binding 2	3.3
AI860822	b	nucleolar antigen Nop52	Rrp1	Pre-rRNA processing	3.2
Y12065	a,b	nucleolar protein hNop56	Nop56	Component of C/D-box snoRNPs	3.3
AI983332	a,b	nucleolar protein Nop5/Nop58	Nop58	Component of C/D-box snoRNPs	2.9
X55504	b	nucleolar protein 1/P120 antigen	Nop2p	Putative methyltransferase	2.6
AA837495	b	nucleolar protein Gar1	Gar1	Component of the H/ACA-box snoRNPs	3.1
L05425	b	nucleolar GTPase	Nog2	Found in pre-60S ribosomal particles	2.0
AJ006591	b	nucleolar cysteine-rich protein		Localisation to the nucleolus, associates with centromeres	2.1
AF023612	b	Dim1p homolog	Dim1p	Adenine-dimethyltransferase	1.8
U59151	b	dyskerin	Cbf5p	Pseudouridine-synthase of H/ACA-box snoRNPs	3.5
D21262	a,b	KIAA0035 (rat Nopp140)	Srp40p	Nucleologenesis and nucleolar integrity	6.5
D25218	a,b	KIAA0112	Rrs1	Regulatory proptein required for ribosome biogenesis	4.6
AI206221	b	KIAA0185	Rrp5	Putative bridging protein, ribosomal RNA processing	3.0
D50914	a,b	KIAA0124 (Bop1)	Erb1p	Ribosomal RNA processing	5.0
AJ001340	b	U3 snoRNP associated protein		Component of snoRNPs	2.1
U78310	b	pescadillo	Nop7	Processing, nuclear export, interacts with ORC	2.6
U59435		cell cycle protein p38–2G4		Proliferation-associated, 38kDa	3.9
D49490		PDIR		Protein disulfide isomerase-related protein	5.0
D21853		KIAA0111	Fal1	Initiation factor, protein biosynthesis, helicase	1.9
U07231	a	G-rich sequence factor-1		RNA-binding	1.9
AW001374	a	prohibitin	Phb1	Unknown	2.2
T96408		bystin		Homologue to drosophila bys	7.9
R20554		glucose regulated protein	Pdi1	Has protein disulfide isomerase activity	1.4
AW023676	a	mortalin-2		Heat shock 70kDa protein 9B	2.3
AW001281	a	LPS-associated protein 1		Heat shock 70kDa protein 8	1.7
AI832665		DKFZP586M0122	Rpa190	Likely ortholog of mouse RNA polymerase 1–4 (194 kDa subunit)	2.8
AI682973	a	eIF4A, isoform 1	Tif2	Binding of mRNA to 40S ribosomal subunit	1.4
W07032	a	ribosomal protein S19		Ribosome biogenesis	2.3
H45858	a	ribosomal protein S17		Ribosome biogenesis	2.0
AW043742	a	ribosomal protein S11		Ribosome biogenesis	2.0
AI037949		ribosomal protein S24		Ribosome biogenesis	3.1
AI553745		hypothetical protein HSPC111		Unknown	4.5
AI989533	b	C2f protein	Mra1	Ribosomal RNA processing	2.2
D29958	a,b	KIAA0116 protein	Rrp45	Exosome complex exonuclease	3.5
AW004842	b	RNA processing factor 1	Rpf1	Ribosome biogenesis	1.5
AI985787	a	hypothetical protein FLJ10439		Contains three WD domains (WD-40 repeat)	4.4
AI417099		WD repeat domain 12 protein	YOR272	Unknown	8.7
AI458823	b	DKFZP564O0463 protein	Sof1	18S rRNA processing	2.2
AI140114		CGI-48 protein	YJL069	Unknown	2.4
D13645	a	KIAA0020 gene	YDR496	Minor histocompatibility antigen HA-8	3.2
AJ007398	b	PBK1 protein	YKR060	Ribosome biogenesis	2.1
AL040968	ab	nucleostemin	Nug1	Putative nucleotide binding protein	4.0
AW008363	b	G protein-binding protein CRFG	Nog1	Essential nucleolar G protein-binding protein	2.7
AI638620		hypothetical protein FLJ14075	YGR145	Unknown	2.4
AA251235		KIAA0007 protein	YDR398	Contains WD domains (WD-40 repeat)	2.9
D80001	a	KIAA0179 gene	Rrp1	Unknown	5.2
U28042	a,b	DEAD/H box polypeptide 10	Hca4	Ribosomal RNA processing	3.8
AI880771	b	WD repeat-containing protein 3	Dip2	U3 snoRNP protein	4.0
U88153		PELP1	Spt7	Plays a permissive role in E2-mediated cell cycle progression	2.2
U49844		ataxia telangiectasia/Rad3 related	Rad3	Cell cycle checkpoint and DNA damage repair	2.0
AL040581	a	MYB binding protein (P160) 1a		Nucleolar protein that binds MYB	2.6
AA194366		DEAD box helicase 97 kDa	DBP10	ATP-dependent RNA helicase	1.8
AF054996	b	U3 snoRNP protein 4 homolog	YNL075	Ribosome biogenesis	2.3
AA877527		CGI-115 protein	YPR143	Unknown	2.5
AA523292	a	surfeit 6		Novel nucleolar protein	2.2

Table 1. *Continued*

Accession number Ho15.19 MycER:		Name	Yeast	Function	Signal ratio
M55015	a,b	nucleolin	Nsr1p	Ribosome biogenesis	1.6
M94287	a,b	Nopp140	Srp40p	Nucleologenesis and nucleolar integrity	2.2
AF069782	b	Nap65	Nop5/58	Component of C/D-box snoRNPs	1.8
AI169617	a,b	EST, KIAA0124 (Bop1)	Erb1p	Ribosomal RNA processing	2.4
AA997726	b	EST, RRP5 homolog	Rrp5	Ribosomal RNA processing	2.2
AI171263	a,b	EST, mouse fibrillarin	Nop1	Component of small nucleolar ribonucleoprotein (snRNP)	2.7
AI104979	a,b	EST, nucleolar protein p40	Ebp2	Ribosomal RNA processing	2.3
AA963703		EST, P38-2G4		Proliferation associated, 38kDa	2.3

a, genes that have been described as c-Myc targets in other screens.
b, genes involved in ribosome biogenesis.

parental cell line EREB2-5 (14) and of Ho15.19MycER cells (Smoxi4) (15) are described elsewhere. Activation of MycER was induced by adding 0.1 μg/ml 4-hydroxy-tamoxifen (Sigma). Where indicated, cells were treated with 30 μM roscovitine (ICN). Human primary fibroblasts were cultivated in early passages in DMEM (Invitrogen) with 15% FCS.

Northern blots

Northern blot analysis was performed as described elsewhere (16). Probes: Myc, cDNA of exon 2 and 3; human Bop1, accession number D50914, PCR fragment from nucleotides 160–899; human Nop56, V12065, nucleotides 199–1226; human fibrillarin, X56597, nucleotides 245–840; Cbf5p, U59151, nucleotides 724–1387; human spermidine synthase, M64231, nucleotides 163–861; human cdk4, M1450, nucleotides 286–487; human GAPDH cDNA; 7SK, X05490, nucleotides 231–330; 5′-ETS, U13369, nucleotides 1–50; rat Bop1, U77415, nucleotides 1951–2246; rat Nop56, BI295000, nucleotides 93–579; rat fibrillarin, AI171263, nucleotides 97–508; rat transferrin receptor, M58040, nucleotides 1504–2491; rat ODC, M19157, nucleotides 453–1618; rat Ssecks, RNU 23146, nucleotides 4–889; rat Gadd45, L32591, nucleotides 19–691; rat GAPDH, NM_017008, nucleotides 21–746.

High-density oligonucleotide arrays

Target cRNA was prepared and hybridised (45°C, 16 h) to the human HG95 chip set and the rat U34 chip set according to the manufacturer's directions (Affymetrix). Arrays were washed and stained using the GeneChip fluidics station 400 and scanned using the Agilent GeneArray scanner. Signals were analysed using Microarray Suite 5.0 software (Affymetrix). Conditional c-Myc induction was performed in three independent experiments. For the expression analysis, P493-6 cells were arrested (see above) and re-stimulated with 10% FCS for 8 h in the presence or absence of c-Myc (control). Total RNA was isolated and prepared for the chip analysis. The Ho15.19MycER cells were grown to confluence to arrest the cells. After 2 days at confluence, MycER was activated by adding tamoxifen for 8 h. Non-induced cells served as a control. The replicates were used to calculate means and standard deviations for the signal values of all probe sets from each cell line and condition. The signal was calculated using the One-Step Tukey's Biweight Estimate. For the comparison analysis the Wilcoxon's signed-rank test was used (for details see Statistical Algorithms Reference Guide, Affymetrix). The *P*-value for the change call 'increase' was set at least by 0.002, for the change call 'decreased' by at least 0.998. The criteria for a differentially expressed gene after c-Myc induction were as follows. Induced target genes must have an 'induced' change call and must be 'present' on the induced array, whereas they can be either present or absent on the un-induced array. Repressed target genes must have a 'decreased' change call and must be 'present' on the un-induced array, whereas they can be either present or absent on the induced array. These criteria were applied to all possible comparisons between the triplicate experiments to determine c-Myc target genes. Because of the highly stringent *P*-value, genes that have a differential expression less than the factor 2 were also included (Table 1).

Nuclear run-ons

Isolation of nuclei, nuclear run-on reaction and hybridisation were carried out as previously described (17). Labelled RNA was hybridised to pan oligonucleotides of the rDNA gene U13369 (18S: nucleotides 4014–4063, 28S: nucleotides 9561–9610, 5.8S: nucleotides 6690–6739), 7SK (X05490: nucleotides 231–330), GAPDH (X01677: nucleotides 351–450) and actin (X00351: nucleotides 151–260) that were bound on a nylon membrane (Hybond N+, Amersham). Signal intensities were analysed with a PhosphoImager (Fuji-BAS 1000).

Pulse–chase labelling with ^{3}H-uridine

Cells were pulsed for 30 min in medium containing 2.5 μCi/ml ^{3}H-uridine (ICN) and chased in medium containing 0.5 mM uridine. RNA was isolated, separated on a 1% formaldehyde agarose gel and transferred to a nylon membrane, which was treated with En^{3}Hance (New England Nuclear) and exposed to Kodak X-Omat AR film. Quantification analysis was performed with the NIH Image programme (http://rsb.info.nih.gov/nih-image/).

RESULTS

c-Myc target genes in the human B-cell line P493-6 and the rat fibroblast line Ho15.19MycER are involved in ribosome biogenesis

To identify genes that are regulated by c-Myc, we analysed the expression profiles of a human B-cell line (P493-6) carrying a

c-*myc* tet-off system and a rat c-*myc* null fibroblast cell line carrying a MYC-ER (Ho15.19MycER). Comparison of cell lines from different species and tissues should assist the identification of conserved c-Myc target genes with general functions. Screens were performed with Affymetrix technology using the human chip set HU95 A-E and the rat chip set RG35 A-C (for details see Materials and Methods). This screen of the human B-cell line P493-6 with ~60 000 gene probes exceeded a previous screen with ~6800 probes (18). The rat cells were screened with a chip set containing ~24 000 gene probes. Many of the identified c-Myc target genes were only present as ESTs on the chips, but the corresponding genes could be identified with the annotations of the Stanford gene bank database (http://genome-www.stanford.edu).

The largest class of genes induced by c-Myc in both cell systems contained genes with nucleolar functions (Table 1). Sixty-four nucleolar genes were induced in P493-6 cells and eight of these genes were also up-regulated in Ho15.19MycER cells. Twenty-six of the target genes identified here have been described as c-Myc target genes before, e.g. fibrillarin, nucleolin, nucleophosmin and others (http://www.myc-cancer-gene.org). Noteworthy, almost all of these genes were also induced by c-Myc in serum starved P493-6 cells (data not shown), where c-Myc readily can induce cell growth (increase in cell size) but fails to induce cell proliferation (5). Many of the yeast homologues of known and new target genes identified here, are suggested to have an important role in processing of rRNA (19). The results of the microarray screens were confirmed for a set of known and new target genes by northern blot analysis. The nucleolar genes Bop1, Nop56, fibrillarin and dyskerin were induced upon c-Myc activation in P493-6 cells with the same kinetics as the previously described direct target gene cdk4 (20) (Fig. 1A). c-Myc-dependent regulation of nucleolar and other target genes was also seen in Ho15.19MycER cells (Fig. 1B).

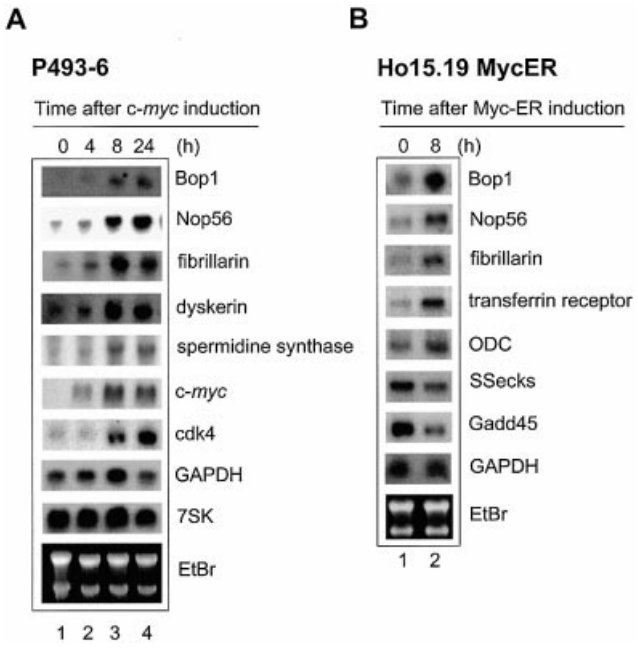

Figure 1. Northern analysis of c-Myc target genes. (**A**) P493-6 cells were arrested for 72 h (0 h) and stimulated with c-Myc for 4, 8 and 24 h. (**B**) The Ho15.19MycER cells were arrested 2 days at confluence, followed by MycER activation for 8 h. Non-induced cells served as a control (0 h). RNAs were hybridised with probes specific for the indicated genes. Spermidine synthase, cdk4, transferrin receptor, ODC, fibrillarin and Gadd45 are known c-Myc target genes and are used for validation of the assay. Ssecks is a new target gene identified in this screen. The housekeeping gene GAPDH and the 7SK gene served as controls. GAPDH showed a small transient increase at 8 h in P493-6 cells. EtBr, ethidium bromide-stained RNA.

c-Myc-dependent production of the 18S and 28S rRNA

A large number of the nucleolar genes listed in Table 1 are directly involved in rRNA processing. To test whether processing of the 47S rRNA precursor in P493-6 cells is regulated by c-Myc, we performed pulse–chase labelling experiments. Cells were starved for 72 h at 0.25% serum in the presence of tet (arrested cells) and were subsequently stimulated for 12 h with 10% serum in the presence or absence of tet. Cells were then pulse-labelled for 30 min with ^{3}H-uridine followed by a chase for indicated time points (Fig. 2). In the absence of tet, high levels of mature 18S and 28S rRNA accumulated during the chase kinetic (Fig. 2A). In contrast, only a little increase of mature forms of rRNA was seen if cells were labelled in the presence of tet. After 6 h chase, the amount of processed 28S rRNA was ~5-fold higher in cells expressing c-*myc* (Fig. 2B). When arrested cells were stimulated only by c-Myc, rRNA processing was almost as efficient as in the presence of c-Myc plus FCS (Fig. 2C). Arrested cells showed no labelling of rRNA (data not shown). Thus, P493-6 cells appear to have a severe defect in production of mature 18S and 28S rRNA when c-Myc is down-regulated by tet. This defect could be due to a decreased processing of the 47S precursor or a reduced transcription rate of rDNA genes.

c-Myc and serum can stimulate production of the 47S rRNA precursor

c-Myc had a strong positive effect on the production of the mature 18S and 28S rRNAs. To investigate the level at which this regulation occurs, we measured the amount of total 47S precursor rRNA by northern analysis and compared it with the amount of radioactively labelled precursor in pulse–chase experiments (Fig. 3A). Arrested cells were stimulated with 10% FCS in the absence or presence of tet for the indicated time points. The 47S precursor was detected with a 5′-ETS leader probe in northern analysis. Since removal of this leader sequence from the 47S precursor is the first step during rRNA processing, only the newly transcribed full-length precursor hybridises to this probe. Therefore, the abundance of the 47S precursor is often used as a measure for transcription of rDNA genes. Surprisingly, the amount of precursor detected with this probe did not significantly change in arrested and stimulated P493-6 cells (Fig. 3A, 5′-ETS).

However, a pulse–chase labelling experiment in P493-6 cells indicated that the turn-over rate of the 47S rRNA precursor is affected by FCS and c-Myc. Arrested cells did not incorporate ^{3}H-uridine into the 47S precursor (Fig. 3A, lanes 1 and 9), whereas significant incorporation of ^{3}H-uridine into the 47S precursor was already detectable 2 h after serum stimulation of P493-6 cells (lanes 2 and 10). Incorporation of ^{3}H-uridine into the 47S precursor was also induced by c-Myc in the absence of FCS (Fig. 2C, lane 3). In conclusion, the data

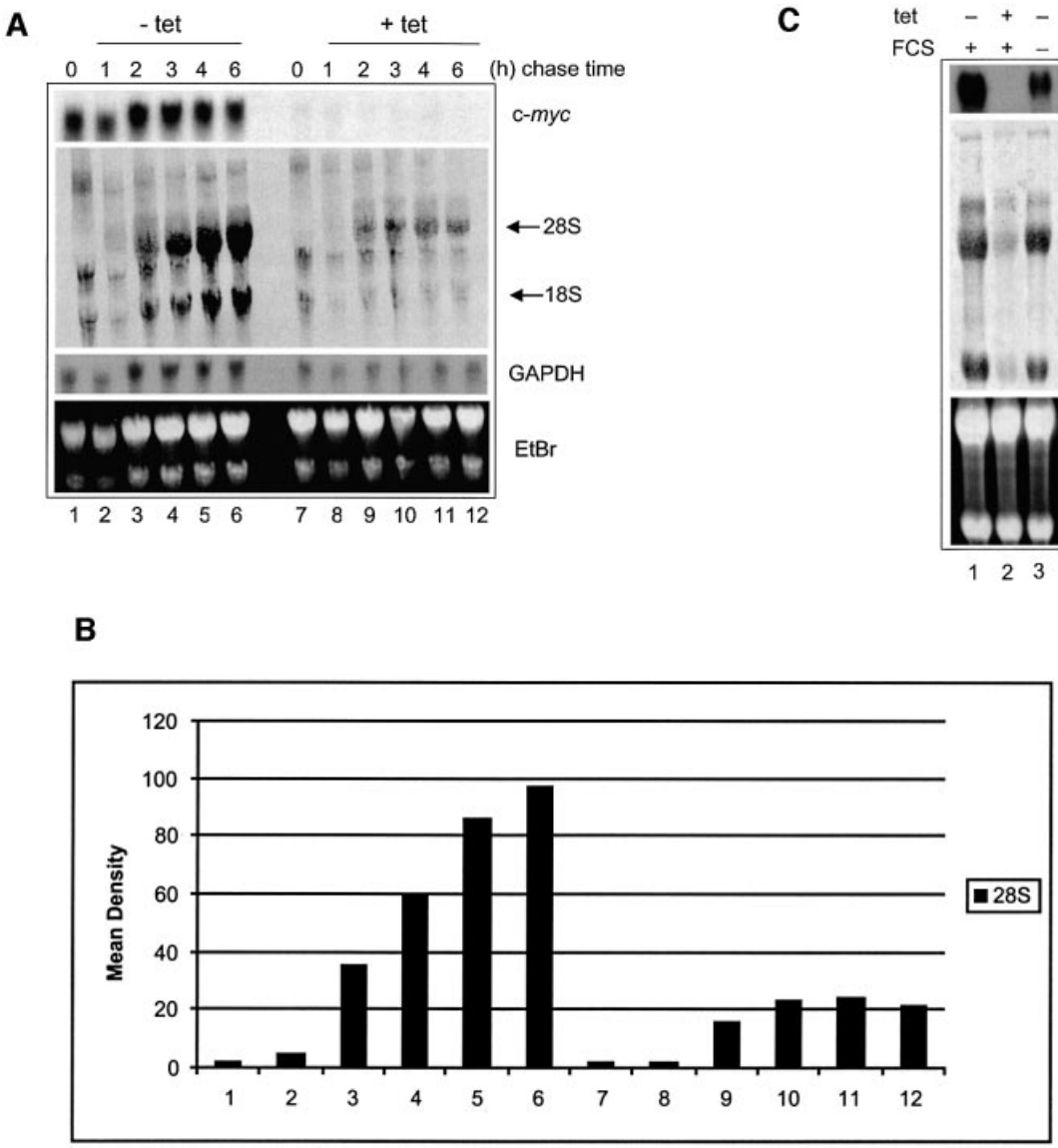

Figure 2. c-Myc-driven rRNA maturation in P493-6 cells. (**A**) Cells were arrested for 72 h and subsequently serum stimulated in the absence or presence of c-Myc. After 12 h, the cells were pulse-labelled with 2.5 µCi/ml ^{3}H-uridine for 30 min and chased for the indicated times. Labelled RNAs were isolated, separated on a 1% agarose formaldehyde gel, transferred to a nylon membrane and visualized by fluorography. The same blot was hybridised with a probe specific for c-*myc*. Results of a representative experiment are shown. (**B**) Quantification of signals of the incorporated ^{3}H-uridine in 28S rRNA. (**C**) Cells were arrested for 72 h, stimulated for 12 h in the presence of c-Myc, FCS or both, pulsed for 30 min with ^{3}H-uridine and chased for 4 h. Signals for *c-myc* were detected by northern analysis.

show that FCS and c-Myc can independently stimulate the synthesis of the 47S rRNA precursor.

As mentioned above, the high abundance of the 47S precursor detected by the 5′-ETS probe in northern analysis was unexpected in un-stimulated P493-6 cells. To investigate whether this observation was specific for P493-6 cells, we tested the parental cell line EREB2-5 (14). EREB2-5 cells are primary B-lymphocytes infected with an EBV mutant expressing a conditional EBNA2-ER. In the absence of oestrogen and serum, EREB2-5 cells enter a quiescent, non-proliferative state. The 47S precursor was not detectable in arrested cells in northern analysis, but was up-regulated in stimulated cells (Fig. 3B, 5′-ETS). Thus, the high level of the 47S precursor was specific for arrested P493-6 cells, but was not observed in quiescent EREB2-5 cells. The high abundance of the 47S rRNA precursor may be due to a leakiness of the tet-regulated c-*myc* construct in P493-6 cells (Discussion).

The density of RNA polymerase I complexes on ribosomal genes is independent of mitogenic stimulation and c-Myc

The pulse–chase labelling experiment above indicated that radiolabel is incorporated into the 47S precursor only after stimulation of cells. We performed nuclear run-on

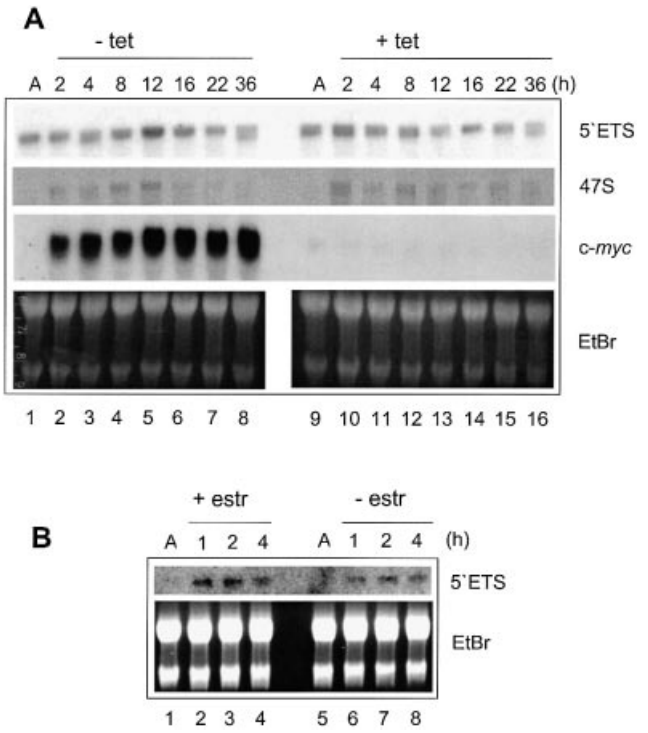

Figure 3. Kinetics of 47S rRNA production. (**A**) P493-6 cells were arrested for 72 h and serum stimulated in the absence or presence of c-Myc for the indicated times. Subsequently, cells were pulse-labelled with ^{3}H-uridine for 30 min and chased for 1 h. The 47S RNAs was visualised by fluorography as described. The same blot was hybridised with a probe specific for the 5′-ETS of the 47 S precursor and c-*myc*. (**B**) Northern blot with the 5′-ETS probe. EREB2-5 cells were depleted for 72 h of FCS and oestrogen (A) and then serum stimulated in the absence or presence of estrogen for the indicated times.

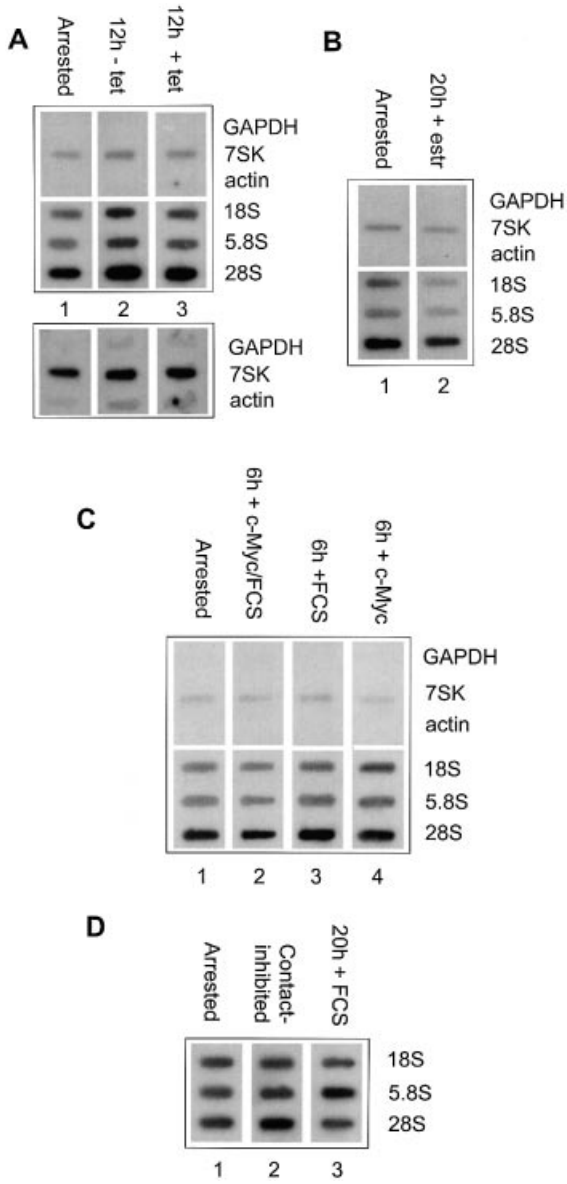

Figure 4. Density of pol I complexes on ribsomal genes. (**A**) Nuclei were prepared from arrested P493-6 cells and from cells 12 h after serum stimulation in the absence or presence of c-Myc. Nuclear run-on reactions were performed. Labelled RNAs were purified and hybridised to probes specific for the indicated genes. A six-times longer exposure is shown in the lower panel. (**B**) Run-on assay in EREB2-5 cells. Arrested cells were stimulated for 20 h with oestrogen and run-on reactions were carried out as described. (**C**) Run-on assay in Ho15.19 MycER cells. Arrested cells were stimulated for 6 h with Myc/FCS, FCS or Myc. (**D**) Human primary fibroblasts were arrested for 72 h in 0.25% FCS. Run-on reactions were carried out in arrested, confluent and serum-stimulated cells.

experiments to measure the density of pol I molecules on rDNA genes before and after stimulation of P493-6 cells. Strong transcription signals were obtained for oligonucleotide probes corresponding to the 5.8S, 18S and 28S rDNA sequence in arrested cells (Fig. 4A, lane 1). These signals increased only slightly if cells were stimulated for 12 h with c-Myc/FCS (lane 2) or FCS alone (lane 3). Signals of the pol II transcribed genes GAPDH and actin could only be detected after six times longer exposure (lower panel). All signals were suppressed >95% when cells were pre-treated for 1 h with 2 µg/ml Actinomycin-D, an inhibitor of transcription (data not shown). The high abundance of pol I on ribosomal genes in arrested cells was unexpected and could be the reason for the high abundance of the 47S precursor observed in these cells. Therefore we also analysed arrested EREB2-5 cells, Ho15.19MycER cells and human primary fibroblast (Fig. 4B–D). In all experiments, we found high levels of pol I complexes on ribosomal genes in arrested cells, which did not significantly increase after stimulation. The results strongly indicate that in arrested cells pol I pauses at multiple positions on ribosomal genes. In isolated nuclei, the paused pol I complexes become activated if the run-on reaction is carried out. The results suggest that transcription of rDNA genes is mainly regulated at the level of rRNA elongation.

Notably, c-Myc has recently been reported to regulate the expression of several pol III-specific genes (21). However, no difference for the transcription signal of the pol III-specific 7SK gene was detected after stimulation with c-Myc in P493-6 and Ho15.19MycER cells (Fig. 4A and C). We could also not detect any changes in the steady-state levels of 7SK RNA after up-regulation of c-Myc (Fig. 1A). Thus, 7SK seems to be an example of a pol III transcribed gene that is not regulated by c-Myc.

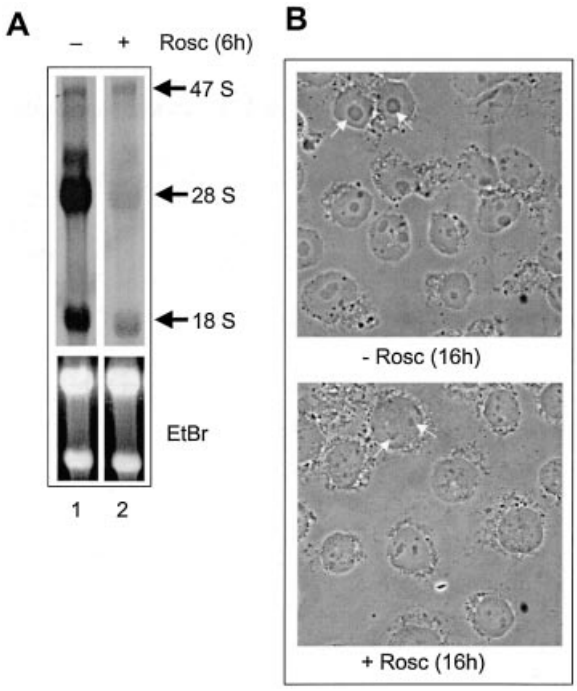

Figure 5. Inhibition of rRNA processing by roscovitine. (**A**) Proliferating P493-6 cells were labelled with ^{3}H-uridine for 6 h in the absence (–) and presence (+) of roscovitine (Rosc). rRNA was analysed as described. (**B**) Proliferating P493-6 cells were cultured in the presence of roscovitine for 16 h. Intact nucleoli in untreated cells and pre-nucleolar bodies after treatment with roscovitine are indicated by arrows.

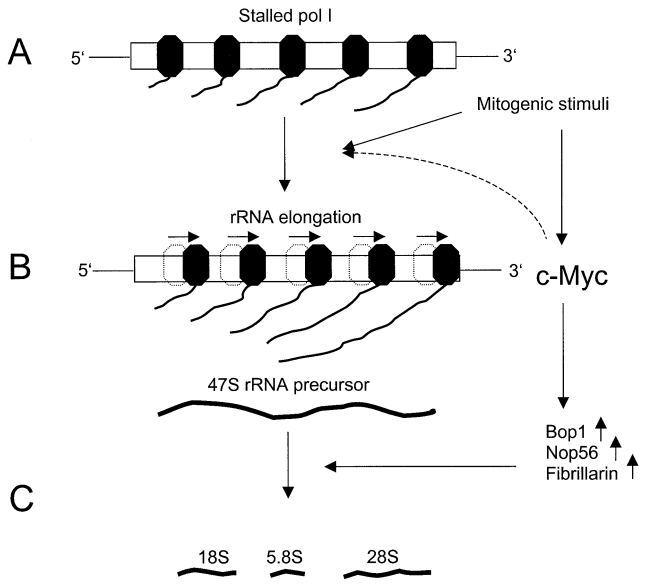

Figure 6. Model of regulation of rRNA processing by c-Myc in P493-6 cells. (**A**) High density of pol I molecules on ribosomal genes in quiescent cells. (**B**) Mitogenic stimulation induces rRNA elongation and production of the 47S rRNA precursor. (**C**) Efficient processing of the 47S rRNA precursor takes place only if c-Myc target genes are up-regulated.

Inhibition of cdk activity blocks processing of rRNA

Processing of the 47S rRNA precursor has recently been shown to require the activity of cdk2 (22). We asked to what extent c-Myc-induced rRNA processing is sensitive to the cdk2 inhibitor roscovitine. rRNA was labelled in pulse–chase experiments in P493-6 cells, and rRNA processing was studied in the presence of roscovitine. Processing of the 47S rRNA was almost entirely blocked by roscovitine after 6 h, while the effect on 47S rRNA production was only minor (Fig. 5A). Roscovitine has recently been reported to induce a rapid disintegration of the interphase nucleolus structure (22). This disintegration was also observed if P493-6 cells were treated with roscovitine (Fig. 5B). We have previously shown that roscovitine cannot inhibit c-Myc-induced growth in P493-6 (5). This previous observation and the data shown here implicate that c-Myc can induce growth in P493-6 cells even if cdk2 activity and *de novo* rRNA synthesis are blocked.

DISCUSSION

The screens carried out in this study clearly underscore the function of c-Myc in the regulation of nucleolar processes. Together, 64 genes encoding proteins with nucleolar localization were identified, 26 of which have been described as c-Myc targets before (Table 1). The majority of the target genes are involved in ribosome biogenesis, in particular in processing of the 47S ribosomal RNA precursor. Processing of rRNA and ribosome biogenesis has been studied in detail in *Saccharomyces cerevisiae*. Unfortunately, understanding these processes in higher eukaryotes, including humans, still lags far behind yeast (10). However, the majority of involved factors identified in yeast, have homologues in humans and other eukaryotes. Several homologues of the c-Myc target genes identified here are involved in the early rRNA processing and maturation steps: Nop5/Nop58 and Nop56 are both components of C/D-box snoRNPs (23). GAR1 is required for pre-rRNA pseudouridylation and processing at early sites (24). Further nucleolar yeast homologues of c-Myc target genes are listed: Nsrp1 (homologous to nucleolin), Nop1p (fibrillarin) and Nop2p (p120) are important factors for ribosome biogenesis. Nop1p is a common component of all C/D-box snoRNPs and is involved in all major post-transcriptional activities in ribosome synthesis, pre-rRNA processing, pre-rRNA modification and ribosome assembly (25). Nop2p is a putative methyltransferase and is required for pre-rRNA processing and 60S ribosome subunit synthesis (26). Pescadillo is the mammalian homologue of yeast Nop7p. Mutation of pescadillo in the mouse results in an arrest at morula stages of development, the nucleoli fail to differentiate and accumulation of ribosomes is inhibited (27). A number of nucleolar c-Myc targets appear to have no known yeast homologue. These genes include Gu-alpha and Gu-beta, two DEAD-box RNA helicases, Nopp34, a binding partner of the nucleolar proliferation marker Ki-67, and several others (Table 1).

The large list of c-Myc target genes with nucleolar functions prompted us to investigate whether c-Myc regulates processing of rRNA. Processing of the 47S rRNA precursor into mature 18S and 28S RNA was efficient in the human B-cell line P493-6 only if c-Myc was present. While the positive effect of c-Myc on processing of rRNA was apparent, the effect on rDNA transcription was less clear. The density of pol I molecules on rDNA genes measured in run-on

experiments was almost unchanged in P493-6 cells regardless of whether cells were arrested or stimulated. This was in contrast with the observation that stimulated, but not arrested cells, incorporated ^{3}H-uridine into the 47S precursor. How can the transcription rate of rDNA genes change without significant alteration in pol I density? An explanation could be that transcription of rDNA genes is mainly regulated at the level of rRNA elongation. In this case, the level of pol I complexes on the rDNA genes remains high, even if the genes are not transcribed. The detection of high pol I density on rDNA genes in arrested cells of parental EREB2-5, as well as in Ho15.19 MycER and human primary fibroblasts, confirmed our assumption that control of rRNA elongation may be a general mechanism in the regulation of rDNA gene transcription in quiescent cells.

Processing of the ribosomal precursor has been suggested to be the major regulatory step of ribosome biogenesis (12). Our results support this notion and suggest c-Myc as an important regulator of rRNA processing. We propose from our data the following model in P493-6 cells. High levels of pol I complexes are constitutively present on rDNA genes. Mitogens can stimulate RNA elongation of these complexes. However, the 47S transcript is efficiently processed to the mature forms only if c-Myc is present and the nucleolar target genes are induced (Fig. 6).

An unexpected finding was the detection of high levels of 47S rRNA precursor in arrested P493-6 cells. This peculiarity was not observed in the parental EREB2-5 cell line. What could cause the difference of 47S precursor abundance in arrested P493-6 and EREB2-5 cells? Careful analysis of P493-6 cells revealed a leakiness of the tet-regulated c-*myc* construct in the presence of tetracycline (data not shown). Low amounts of c-Myc may already be sufficient for increased transcription and/or stabilization of the 47S precursor but fail to induce the genes for the processing machinery. This suggests that the sole presence of the 47S precursor is not sufficient to induce its own processing. Recently, Amati and co-workers defined high and low affinity targets for genomic c-Myc binding sites (28). It is possible that transcription of rDNA genes depends on binding of c-Myc to high-affinity sites, whereas processing of the rRNA precursor depends on genes activated by binding of c-Myc to low-affinity sites.

Processing of the 47S rRNA precursor requires stoichiometrical amounts of ribosomal protein (rp). Since there are only a few rp mRNAs up-regulated after c-Myc induction (Table 1), one could speculate that the increased need of rp is met by enhanced translation via the 5′ terminal oligopyrimidine tract (5′TOP) of the rp mRNA (29). It will be interesting to determine if c-Myc contributes to this selective translational control of rp mRNAs.

As shown recently, processing of the 47S rRNA precursor and formation of nucleoli depend on cdk activity (22). In P493-6 cells, roscovitine rapidly blocks processing of rRNA and leads to disaggregation of nucleoli. As shown earlier, c-Myc induces growth in P493-6 cells in the presence of roscovitine (5). This growth programme operates uncoupled from cdk2 activity, cell cycle entry and as shown in this study, uncoupled from synthesis of new ribosomes. Growth in the absence of ribosome biogenesis has also been shown in the liver-specific conditional knockout of the ribosomal S6 gene that resulted in decreased ribosome biogenesis, whereas total cellular translation was unaffected. However, after partial hepatectomy, the liver cells showed a defect in proliferation that occurred concomitantly with a decrease in cyclin E expression. Thus, inhibition of ribosome biogenesis blocks S-phase entry but not necessarily cell growth (30).

The observations in mouse liver cells are in line with the model that cell-cycle activation by c-Myc induces and requires the production of new ribosomes. This implies that cells have implemented two growth programmes: (i) a programme for increasing cell size characterised by increased translation without increasing the number of ribosomes, and (ii) a programme leading to cell division characterised by increased translation coupled to increased cdk activity and ribosome biogenesis. We propose that the latter programme is strongly c-Myc dependent.

c-Myc induced ribosome biogenesis in cell cycle and growth control

If cdk activation controls ribosome biogenesis, does ribosome biogenesis, vice versa, control cell cycle progression? Activation of c-Myc does not only induce cdk activity, it also induces expression of Ink4a/ARF, leading to p53 accumulation and cell cycle arrest. The activation of p53 by c-Myc could reflect a role of p53 in growth control. p53 may inhibit cell cycle progression in c-Myc stimulated cells as long as synthesis of new ribosomes has not occurred. We, and others, have identified Bop1 as a c-Myc target gene. A dominant negative mutant of Bop1 that blocks rRNA processing has recently been shown to block G1 progression in a p53-dependent manner, suggesting that p53 controls G1 progression in normal cells dependent on the proper processing of ribosomal RNA (31). Recently, ARF has also been reported to regulate rRNA processing in a p53 dependent manner (32).

The involvement of deregulated ribosome biogenesis in the development of cancer has recently been reviewed (33). It has long been known by pathologists that hypertrophy of the nucleolus is a characteristic cytological feature of cancer cells (34) but only recently has it been used as a prognostic factor (35). c-Myc, which is over-expressed in many malignancies, induces ribosome biogenesis and protein synthesis by up-regulating ribosomal and nucleolar proteins, translation factors and as shown here, the efficiency of rRNA processing. This regulation could be an important mechanism by which c-Myc induces growth and initiates tumorigenesis. Furthermore, specific inhibition of rRNA processing could be a targeted approach to interfere with downstream effects of an oncogenic c-Myc in various human malignancies. The anti-cancer drug doxorubicin has recently been reported to bind the nucleolar protein Nopp140 (36). It will be interesting to investigate whether doxorubicin counteracts c-Myc function in rRNA processing.

ACKNOWLEDGEMENTS

We thank J. Sedivy for c-*myc* knockout cells, U. Bär for excellent technical advice and S. Humme and A. Korff for critical reading the manuscript. This work was supported by the DFG (EI 216/8-1) and Fonds der Chemischen Industrie.

REFERENCES

1. Henriksson,M. and Luscher,B. (1996) Proteins of the Myc network: essential regulators of cell growth and differentiation. *Adv. Cancer Res.*, **68**, 109–182.
2. Cole,M.D. and McMahon,S.B. (1999) The Myc oncoprotein: a critical evaluation of transactivation and target gene regulation. *Oncogene*, **18**, 2916–2924.
3. Oster,S.K., Ho,C.S., Soucie,E.L. and Penn,L.Z. (2002) The myc oncogene: MarvelouslY Complex. *Adv. Cancer Res.*, **84**, 81–154.
4. Iritani,B.M. and Eisenman,R.N. (1999) c-Myc enhances protein synthesis and cell size during B lymphocyte development. *Proc. Natl Acad. Sci. USA*, **96**, 13180–13185.
5. Schuhmacher,M., Staege,M.S., Pajic,A., Polack,A., Weidle,U.H., Bornkamm,G.W., Eick,D. and Kohlhuber,F. (1999) Control of cell growth by c-Myc in the absence of cell division. *Curr. Biol.*, **9**, 1255–1258.
6. Kim,S., Li,Q., Dang,C.V. and Lee,L.A. (2000) Induction of ribosomal genes and hepatocyte hypertrophy by adenovirus-mediated expression of c-Myc *in vivo*. *Proc. Natl Acad. Sci. USA*, **97**, 11198–11202.
7. Trumpp,A., Refaeli,Y., Oskarsson,T., Gasser,S., Murphy,M., Martin,G.R. and Bishop,J.M. (2001) c-Myc regulates mammalian body size by controlling cell number but not cell size. *Nature*, **414**, 768–773.
8. Johnston,L.A., Prober,D.A., Edgar,B.A., Eisenman,R.N. and Gallant,P. (1999) *Drosophila* myc regulates cellular growth during development. *Cell*, **98**, 779–790.
9. O'Connell,B.C., Cheung,A.F., Simkevich,C.P., Tam,W., Ren,X., Mateyak,M.K. and Sedivy,J.M. (2003) A large scale genetic analysis of c-Myc-regulated gene expression patterns. *J. Biol. Chem.*, **278**, 12563–12573.
10. Fatica,A. and Tollervey,D. (2002) Making ribosomes. *Curr. Opin. Cell Biol.*, **14**, 313–318.
11. Grummt,I. (1999) Regulation of mammalian ribosomal gene transcription by RNA polymerase I. *Prog. Nucleic Acid Res. Mol. Biol.*, **62**, 109–154.
12. Eichler,D.C. and Craig,N. (1994) Processing of eukaryotic ribosomal RNA. *Prog. Nucleic Acid Res. Mol. Biol.*, **49**, 197–239.
13. Dudov,K.P. and Dabeva,M.D. (1983) Post-transcriptional regulation of ribosome formation in the nucleus of regenerating rat liver. *Biochem. J.*, **210**, 183–192.
14. Kempkes,B., Spitkovsky,D., Jansen-Durr,P., Ellwart,J.W., Kremmer,E., Delecluse,H.J., Rottenberger,C., Bornkamm,G.W. and Hammerschmidt,W. (1995) B-cell proliferation and induction of early G1-regulating proteins by Epstein–Barr virus mutants conditional for EBNA2. *EMBO J.*, **14**, 88–96.
15. Holzel,M., Kohlhuber,F., Schlosser,I., Holzel,D., Luscher,B. and Eick,D. (2001) Myc/Max/Mad regulate the frequency but not the duration of productive cell cycles. *EMBO Rep.*, **2**, 1125–1132.
16. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.
17. Meininghaus,M., Chapman,R.D., Horndasch,M. and Eick,D. (2000) Conditional expression of RNA polymerase II in mammalian cells. Deletion of the carboxyl-terminal domain of the large subunit affects early steps in transcription. *J. Biol. Chem.*, **275**, 24375–24382.
18. Schuhmacher,M., Kohlhuber,F., Holzel,M., Kaiser,C., Burtscher,H., Jarsch,M., Bornkamm,G.W., Laux,G., Polack,A., Weidle,U.H. *et al.* (2001) The transcriptional program of a human B cell line in response to Myc. *Nucleic Acids Res.*, **29**, 397–406.
19. Kressler,D., Linder,P. and de La Cruz,J. (1999) Protein trans-acting factors involved in ribosome biogenesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **19**, 7897–7912.
20. Hermeking,H., Rago,C., Schuhmacher,M., Li,Q., Barrett,J.F., Obaya,A.J., O'Connell,B.C., Mateyak,M.K., Tam,W., Kohlhuber,F. *et al.* (2000) Identification of CDK4 as a target of c-MYC. *Proc. Natl Acad. Sci. USA*, **97**, 2229–2234.
21. Gomez-Roman,N., Grandori,C., Eisenman,R.N. and White,R.J. (2003) Direct activation of RNA polymerase III transcription by c-Myc. *Nature*, **421**, 290–294.
22. Sirri,V., Hernandez-Verdun,D. and Roussel,P. (2002) Cyclin-dependent kinases govern formation and maintenance of the nucleolus. *J. Cell Biol.*, **156**, 969–981.
23. Galardi,S., Fatica,A., Bachi,A., Scaloni,A., Presutti,C. and Bozzoni,I. (2002) Purified box C/D snoRNPs are able to reproduce site-specific 2′-O-methylation of target RNA *in vitro*. *Mol. Cell. Biol.*, **22**, 6663–6668.
24. Girard,J.P., Lehtonen,H., Caizergues-Ferrer,M., Amalric,F., Tollervey,D. and Lapeyre,B. (1992) GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. *EMBO J.*, **11**, 673–682.
25. Tollervey,D., Lehtonen,H., Jansen,R., Kern,H. and Hurt,E.C. (1993) Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation and ribosome assembly. *Cell*, **72**, 443–457.
26. Hong,B., Wu,K., Brockenbrough,J.S., Wu,P. and Aris,J.P. (2001) Temperature sensitive nop2 alleles defective in synthesis of 25S rRNA and large ribosomal subunits in *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **29**, 2927–2937.
27. Lerch-Gaggl,A., Haque,J., Li,J., Ning,G., Traktman,P. and Duncan,S.A. (2002) Pescadillo is essential for nucleolar assembly, ribosome biogenesis and mammalian cell proliferation. *J. Biol. Chem.*, **277**, 45347–45355.
28. Fernandez,P.C., Frank,S.R., Wang,L., Schroeder,M., Liu,S., Greene,J., Cocito,A. and Amati,B. (2003) Genomic targets of the human c-Myc protein. *Genes Dev.*, **17**, 1115–1129.
29. Avni,D., Shama,S., Loreni,F. and Meyuhas,O. (1994) Vertebrate mRNAs with a 5′-terminal pyrimidine tract are candidates for translational repression in quiescent cells: characterization of the translational cis-regulatory element. *Mol. Cell. Biol.*, **14**, 3822–3833.
30. Volarevic,S., Stewart,M.J., Ledermann,B., Zilberman,F., Terracciano,L., Montini,E., Grompe,M., Kozma,S.C. and Thomas,G. (2000) Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. *Science*, **288**, 2045–2047.
31. Pestov,D.G., Strezoska,Z. and Lau,L.F. (2001) Evidence of p53-dependent cross-talk between ribosome biogenesis and the cell cycle: effects of nucleolar protein Bop1 on G(1)/S transition. *Mol. Cell. Biol.*, **21**, 4246–4255.
32. Sugimoto,M., Kuo,M.L., Roussel,M.F. and Sherr,C.J. (2003) Nucleolar arf tumor suppressor inhibits ribosomal RNA processing. *Mol. Cell*, **11**, 415–424.
33. Ruggero,D. and Pandolfi,P.P. (2003) Does the ribosome translate cancer? *Nature Rev. Cancer*, **3**, 179–192.
34. Pianese,G. (1896) Beitrag zur Histologie und Aetiologie der Carcinoma. Histologische und experimentelle Untersuchungen. *Beitr. Pathol. Anat. Allg Pathol.*, **142**, 1–193.
35. Derenzini,M., Trere,D., Pession,A., Govoni,M., Sirri,V. and Chieco,P. (2000) Nucleolar size indicates the rapidity of cell proliferation in cancer tissues. *J. Pathol.*, **191**, 181–186.
36. Jin,Y., Yu,J. and Yu,Y.G. (2002) Identification of hNopp140 as a binding partner for doxorubicin with a phage display cloning method. *Chem. Biol.*, **9**, 157–162.
37. Andersen,J.S., Lyon,C.E., Fox,A.H., Leung,A.K., Lam,Y.W., Steen,H., Mann,M. and Lamond,A.I. (2002) Directed proteomic analysis of the human nucleolus. *Curr. Biol.*, **12**, 1–11.