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Regulation of P120 mRNA Levels During Lymphocyte Stimulation: Evidence That the P120 Gene Shares Properties With Early and Late Genes

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Abstract P120 is a growth-regulated nucleolar protein, the expression of which is required for G_1- to S-phase transition in lymphocytes. P120 appears to be involved in ribosomal biogenesis presumptively through its putative role as a rRNA methyltransferase. To better understand the role of P120 in cell cycle progression, we examined the regulation of the P120 gene in resting lymphocytes and in mitogen-stimulated lymphocytes as they progress from G_1-phase toward S-phase. P120 mRNA was detected after the immediate early gene *c-fos* and persisted as the cells approached S-phase. A decrease in P120 mRNA coincided with the expression of histone H3 mRNA. The level of P120 mRNA increased as cells proceeded through G_1-phase, and this increase was attributed to a more than threefold increase in the P120 transcription rate and an increase in P120 mRNA stability. The P120 gene is transcribed in resting lymphocytes, although the steady-state level of P120 is small or nonexistent. P120 mRNA accumulates in resting cells in the presence of the protein synthesis inhibitor cycloheximide. Furthermore, the steady-state level of P120 mRNA increases in the presence of cycloheximide after PHA-stimulation; this level does not increase in cells not treated with this protein synthesis inhibitor. The presence of cycloheximide increases both the transcription rate of the P120 gene and the stability of P120 mRNA. These studies indicate that P120 expression is cell cycle regulated in a complex manner and that the P120 gene has properties of both early and late genes. This time ordered regulation for P120 expression may represent a necessary step for the cell cycle associated increase in ribosomal biogenesis that is required for G_1- to S-phase transition.

Key words: nucleolar protein, rRNA, G_1-phase

The nucleolus is highly responsive to growth stimuli [Busch and Smetana, 1970], and there is a close relationship between the rate of cell growth and ribosomal biogenesis [Maalo and Kjeldgaard, 1966; Nomura et al., 1984]. During the cell cycle there is a coordinated regulation between increased RNA polymerase I activity, increased synthesis of ribosomal precursors, and mitosis [Halleck et al., 1989]. An early observation distinguishing normal cells from cancerous cells was that cancer cells have a nucleolus that is very pleomorphic and hyperactive [Busch and Smetana, 1970]. This observation led researchers to attempt to identify cancer-associated or growth-related proteins using monoclonal antibodies against the nucleoli of tumor cells. These studies led to the discovery of the novel 120 kDa nucleolar protein P120 [Freeman et al., 1988]. The P120 protein was detected in a variety of different cancer tissues and in normal proliferating cells including mitogen-stimulated lymphocytes, but not in resting peripheral blood lymphocytes or in many non-proliferating tissues [Freeman et al., 1988; Freeman et al., 1990].

cDNAs and genomic clones specific for the P120 gene have been isolated, cloned, and sequenced [Fonagy et al., 1989; Larson et al., 1990]. The P120 protein is localized to a novel microfibrillar structure of the nucleolar matrix [Ochs et al., 1988]. This structure contains 25 to 30 peptides that are associated with pre-ribosomal particles, but could be distinguished from them by resistance to RNAse [Freeman et al., 1990]. Retrospective and prospective studies of breast cancer tissues [Freeman et al., 1991; McGrath et al., 1994] indicate that the level of P120 expression is directly correlated with pa-

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tient survival and disease recurrence. P120 expression also correlates with the S-phase fraction; tumors with high S-phase fractions are more likely to have detectable levels of P120 [McGrath et al., 1994]. These findings suggest that the expression level of P120 may relate directly to the proliferative properties of the cell. To further examine this possibility, the proliferative potential and P120 expression level was compared in seven different breast cancer cell lines [Fonagy et al., 1994]. The cell lines could be sub-grouped according to their growth properties: cell lines with a rapid growth rate, those with an intermediate growth rate, and those with a slow growth rate. The levels of P120 protein and mRNA expression were shown to correlate highly with growth rate and percentage of S-phase cells. A cell line from each group was treated with P120 antisense oligodeoxynucleotides, and it was determined that decreasing P120 expression in this manner caused a corresponding decrease in proliferation rate.

P120 protein levels are absent or very low in quiescent fibroblasts, and present at higher levels in dividing, non-transformed fibroblasts. Transformed fibroblasts have 15 to 60 times more P120 protein than do non-transformed fibroblasts [Hazelwood et al., 1989]. In normal and transformed fibroblasts the P120 protein has been shown to be cell cycle regulated [Fonagy et al., 1992]. The protein is expressed in early- to mid-G_1 phase of the cell cycle and peak expression occurred at S-phase. Resting lymphocytes that have been stimulated with phytohemagglutinin (PHA) also show increased levels of P120 protein [Jhaing et al., 1990].

The expression of P120 is necessary for normal cell-cycle progression of lymphocytes [Fonagy et al., 1992]. Treatment of PHA-stimulated lymphocytes with P120 antisense oligodeoxynucleotides inhibits the expression of P120 mRNA and protein and growth arrest in these cells. The antisense-treated cells are able to enter the cell cycle from the G_0 resting phase but are unable to enter S-phase. These findings suggest that P120 may play a role in G_1- to S-phase traversal. P120 is required for proper ribosomal biogenesis in lymphocytes [Wilson et al., 1993] presumably by its role as a methyltransferase in methylating rRNA [Koonin, 1994].

The purpose of the present study was to further determine the regulation of P120 during entry of cells into the cell cycle. Mitogen-stimulated peripheral blood lymphocytes provided a convenient model to study the entry of quiescent cells into the cell cycle. Using this model we examined P120 protein and mRNA expression as the cells entered the G_1 phase of the cell cycle and progressed toward S phase. These studies indicate that P120 has properties of both immediate early and late genes. This complex regulation may be required for the up-regulation of nucleolar function required for cells to progress through the cell cycle.

MATERIALS AND METHODS

Cell Culture

Fresh buffy coats were obtained from the Central Kentucky Blood Center. Peripheral blood lymphocytes (PBL) were purified from the buffy coats using Ficoll-Paque (Pharmacia, Piscataway, NJ) density centrifugation. Cells were plated at a concentration of 3×10^6 cells/ml in RPMI 1640 medium containing 10% FBS, 1% Pen/Strep. Harvested lymphocytes were treated as indicated. Every unit of buffy coats obtained represented one experimental group. Samples from several units (at least 3) were pooled in order to obtain an average sample for the Northern blot analyses, with respect to response level from the mitogen and/or other drugs used. Phytohemagglutinin (PHA) (Sigma, St. Louis, MO) was added to a final concentration of 5 μg/ml where indicated.

Treatment

Actinomycin D, a RNA synthesis inhibitor, (Gibco BRL, Gaithersburg, MD) was added to a final concentration of 5 μg/ml and cycloheximide, a protein synthesis inhibitor, (Sigma, St. Louis, MO), to a final concentration of 10 μg/ml.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Cells were harvested by scraping the flask with a cell scraper, washed, and resuspended in Laemmli electrophoresis solubilization buffer [Laimmli, 1970], at a concentration of 4×10^6 cells/ml, followed by incubation at 100°C for 3 min. Cell lysates were electrophoresed in a 7.5% polyacrylamide gel containing 0.1% SDS as described by Takacs, 1979. Silver staining was done essentially as previously described [Oakley, 1980].

Western blot analysis was performed using the modified method of Towbin et al. [1979]. Briefly, the proteins on the gel were transferred

to a 0.45 mm nitrocellulose filter (Schleicher and Schuell, Keene, NH) by electrophoresis over night at 52 mA. Non-specific binding sites on the membrane were blocked by incubation with 10% chicken serum, 3% bovine serum albumin in tris buffered saline (TBS). The filters were incubated with P120 mAb for 1 hr at 1:1,000 dilution in PBS, and then washed three times for 20 min each with TBS containing 0.5% Tween 20. The P120 mAb was detected with alkaline phosphatase conjugated antimouse Ig, and the color was developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrasodium substrates (Protoblot Immunoblotting System, Promega Corporation, Madison, WI).

Flow Cytometry

Lymphocytes were harvested by trypsinization following the treatment indicated and analyzed for DNA content. We processed 10^6 cells/tube as follows; all centrifugation was performed for 5 min at a speed of 500*g*. Pelleted cells were first incubated with 1 ml of a solution of 20 μg/ml lysophosphatidyl choline in 1% paraformaldehyde for 2 min at room temperature. The cells were then subjected to centrifugation at room temperature and resuspensed in 1 ml of cold (−10°C) absolute methanol. After incubation on ice for 10 min, cells were centrifuged and resuspended in 1 ml of 0.1% Nonidet P-40 (NP-40) and incubated for 5 min (on ice). After a final wash in PBS, the cells were resuspended in Coulter™ DNA-prep stain for analysis of cellular DNA. Labeled cells were analyzed on FACStar flow cytometer (Bectin-Dickson, San Jose, CA) equipped with an argon ion laser. PI emission was measured through a 575/26 filter. Cells were gated on a forward vs. side scatter cytometer gate and 10,000 events were collected for analysis. Overlap merge diagrams and overlap calculations of histograms were achieved by LYSIS II software (Becton-Dickson).

Northern Blot Analysis

Total RNA was prepared from cells by a guanidinium isothiocyanate lysis and subsequent CsCl gradient ultracentrifugation (Sorvall RC 70) (Chirgwin et al., 1979). The RNA pellet was dissolved in a pH 7.4 buffer of 10 mM Tris-HCl, containing 1 mM EDTA and 0.1% SDS and then RNA was extracted with phenol/$CHCl_3$/isoamylalcohol (25:24:1) by volume and precipitated with 2.5 volumes of ethanol and 0.1 volume of 3M sodium acetate, pH 5.2. The amount of RNA was determined spectrophotometrically at 260 nm. RNA was fractionated through 2.2M formaldehyde/1.2% agarose gels and then blotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). The filters were baked and subsequently prehybridized, hybridized in buffer containing 50% formamide, and washed according to standard procedures [Sambrook et al., 1989]. Northern blots were repeated at least three times and a representative experiment was reported.

Radioactive probes were prepared by the random priming method [Feinberg and Vogelstein, 1983] with a kit from Boehringer Mannheim (Indianapolis, IN) using [alpha-^{32}P]deoxycytidine-5′ triphosphate, tetratriethyl-ammonium salt (specific radioactivity: 3,000 Ci/mmol; ICN Biomedical, Inc.). The 1.8 kb EcoRI fragment of P120 cDNA [Fonagy et al., 1989] was used for the P120 probe. The 1.3 kb NaeI fragment of *c-fos* cDNA was used. The entire plasmid pBR322histone H3 was labeled and used. To check for equal loading of the RNA gels, the blots were reprobed with a fragment of human 18S ribosomal DNA [Subrahmanyam et al., 1982].

Densitometry

Western blots, Northern blots, and nuclear runoff analyses were scanned directly by a Bio Image, Visage 2000 (Millipore Corporation) analytical instrument. Whole band computer analysis was done and the intensity of the bands reported.

Nuclear Runoff Analysis

Runoff analysis was performed as described by Yuan and Tucker, 1984. Each runoff experiment was carried out on one unit of PBL and was repeated at least three times. Representative experiments are reported. Briefly, cells were incubated in the media indicated and then harvested by trypsinization. Cells were then washed, counted, and cell numbers equalized. Nuclei were isolated using isotonic shock and then counted and also equalized so that the numbers of nuclei to be labeled would be the same for each condition. ^{32}P-UTP (specific radioactivity: 3,000 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA) was added to the nuclei and allowed to incorporate into nascent mRNA. Total RNA was then isolated using a cesium chloride gradient and the labeled RNA was then hybridized to nitrocellulose filters (Schleicher and Schuell,

Keene, NH) onto which the different plasmids of choice had been bound. The plasmids used were pMAM, pMAMp120 (containing the entire 3.1 kb P120 insert), and pBR322histone H3. The filters were then washed, placed onto film for 1–3 days, and then developed.

RESULTS

Monitoring of Cell Cycle Progression

The cell-cycle progression of resting and PHA-stimulated lymphocytes was monitored by flow cytometric analysis of DNA content. The cell cycle distribution of cells at various times after PHA stimulation is shown as the mean of three separate experiments in Table I. Most of the non-PHA-stimulated lymphocytes were in G_0/G_1 phase (98%). By 48 h of PHA stimulation, approximately 38% of the cells were in S-phase. After 60 h of PHA stimulation, there was an approximate equal distribution of cells in S phase (27%) and in G_2/M-phase (24%). Therefore, the lymphocytes enter the cell cycle from G_0 in a semi-synchronous fashion with the peak of S phase cells occurring after 24 h and prior to 60 h of stimulation.

Kinetics of P120 Protein Expression in Resting and Mitogen-Stimulated Lymphocytes

The steady-state level of P120 protein was determined in resting lymphocytes and in lymphocytes after PHA stimulation. Western blot analysis (Fig. 1) showed that the P120 protein was not detected in resting lymphocytes plated in serum-containing medium without PHA. P120 protein was detected in G_1-phase by 16 h of PHA stimulation; the level of P120 protein level continued to increase in amounts at 24 h and 48 h.

Transcriptional Regulation of P120 During the Cell Cycle

Nuclear runoff analysis was used to determine whether transcriptional regulation played a role in the cell cycle related expression of P120. Figure 2A shows a typical nuclear runoff profile in resting lymphocytes and in lymphocytes at various times after stimulated with PHA. Figure 2B shows densitometric scans of the nuclear run-off blots from Figure 2A. The transcription rate of histone H3 was examined because it is transcriptionally up-regulated during S-phase and therefore provides a good marker for DNA synthesis. The pMAM plasmid alone, lacking the P120 cDNA insert, was used as a control and showed little or no binding (data not shown). This analysis suggests that P120 is transcribed in resting lymphocytes and that the transcription rate of the P120 gene increases more than threefold as cells proceed through G_1 (Fig. 2B). The dramatic increase in P120 transcription rate was concurrent with the increased rate of histone H3 transcription. Histone H3 expression was not detected in resting cells, suggesting that the expression of P120 mRNA in resting cells is not due to a sub-population of cycling cells. Thus, the increased expression of P120 as lymphocytes approach the G_1/S-phase boarder is at least partially transcriptionally regulated.

Role of P120 mRNA Stability in Cell Cycle Expression of P120

Actinomycin D treatment, which inhibits RNA synthesis, was used to determine whether mRNA stability plays a role in P120 regulation as cells progress through the cell cycle. Figure 3 shows a representative Northern blot and densitometric scan of this blot from lymphocytes stimulated with PHA for 2 h and 48 h and subsequently

TABLE I. Flow Cytometric Analysis of PHA-Stimulated Lymphocytes*

	Time (h)				
	0	12	24	48	60
% G_0–G_1 phase	98 ± 1.5	92 ± 1.2	81 ± 24	50 ± 6.6	49 ± 6.1
% S Phase	1 ± 1.2	3 ± 1.5	14 ± 18	38 ± 5.5	27 ± 3.2
% G_2-M phase	1 ± 1	5 ± 1.7	5 ± 5.5	12 ± 12	24 ± 2.5

*DNA content determination by flow cytometric analysis to determine the cell cycle phase of PHA stimulated lymphocytes. Lymphocytes were plated in medium containing 5 μg/ml PHA for the length of time indicated and then analyzed for DNA content by flow cytometry, as described in Materials and Methods. Results are listed as the mean of three separate experiments ± the standard deviation.

treated with actinomycin D. Lymphocytes stimulated for 2 h were in early G_1-phase, and at 48 h the cell population was enriched in S-phase cells (Table I). Densitometric scans of the Northern blots indicated that the P120 mRNA in lymphocytes stimulated for 48 h was more stable than the P120 transcript present in cells stimulated for 2 h (Fig. 3B; compare time points 1 h through 2 h). A transient increase in P120 mRNA was observed at 30 min after treatment with actinomycin D (Fig. 3A, lane 2). The findings of this experiment were representative of a typical profile seen in three separate experiments using different lymphocytes.

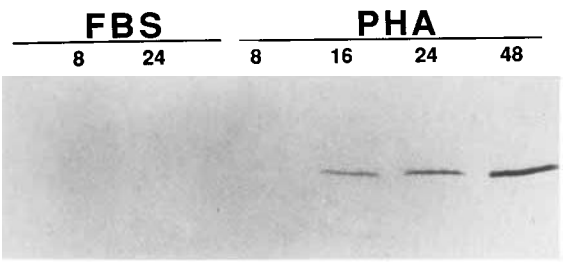

Fig. 1. Western blot analysis of P120 protein expression in PHA-stimulated lymphocytes. Peripheral blood lymphocytes (PBL) were plated in RPMI medium with 10% FBS for 8 h (*lane 1*); PBL plated in 10% FBS for 24 h (*lane 2*); PBL plated in medium containing 5 μg/ml PHA for 8, 16, 24, and 48 h (*lanes 3–6*). The P120 antigen was detected by immunostaining with the P120 mAb.

Protein Synthesis Is Not Required for Induction of P120 mRNA Synthesis

To determine whether protein synthesis was required for P120 synthesis after stimulation, lymphocytes were treated with cycloheximide and PHA (Fig. 4). Figure 4A shows the Northern blot analysis and Figure 4B shows the graphic representation of the densitometric scan of the Northern blot. The Northern blot was probed for P120 and *c-fos;* and as a loading control the blot was also probed for 18S rRNA. In the presence of cycloheximide, P120 showed an accumulation of mRNA (compare Fig. 4A, lanes 3, 5, 7, and 9 with lanes 2, 4, 6, and 8), similar to what has been reported for the immediate early gene product, *c-fos* [Greenberg and Ziff, 1984]. Therefore, we probed the blot for *c-fos*. As with P120, *c-fos* showed an accumulation of mRNA in the presence of cycloheximide (compare Figure 4A, lanes 3, 5, 7, and 9 with lanes 2, 4, 6, and 8). Thus P120, like immediate early genes, does not require protein synthesis for induction. However, unlike the immediate

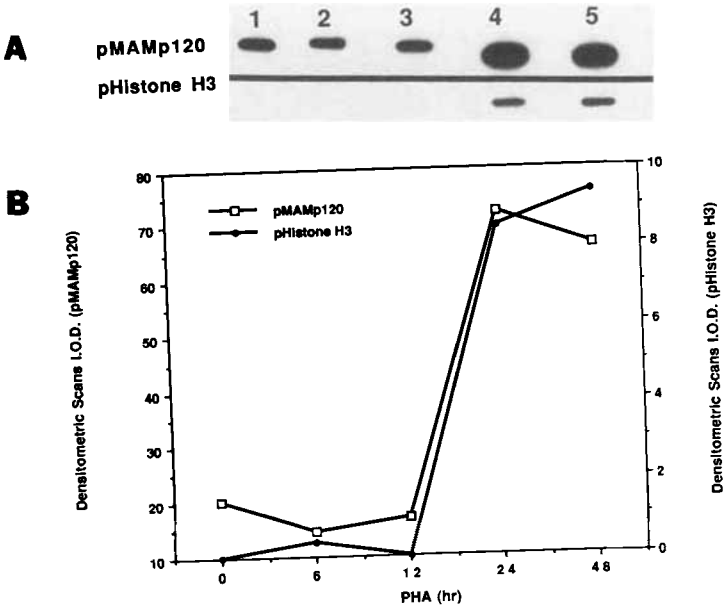

Fig. 2. Transcriptional rates for P120 and histone H3 were examined using nuclear runoff analysis of PHA-stimulated lymphocytes. Five milligrams of the plasmids, pMAMp120 and pBR322Histone H3 (pHistone H3), were hybridized onto the nitrocellulose prior to incubation with the radiolabeled RNA from the treated samples. Lymphocytes were stimulated with PHA (5 μg/ml) for various lengths of time. Following incubation, cells were harvested and their nuclei purified and used in the runoff analyses. **A:** Slot blot, resting lymphocytes (*lane 1*), 6 h (*lane 2*), 12 h (*lane 3*), 24 h (*lane 4*), 48 h PHA stimulation (*lane 5*). **B:** Densitometric scans of slot blots from A.

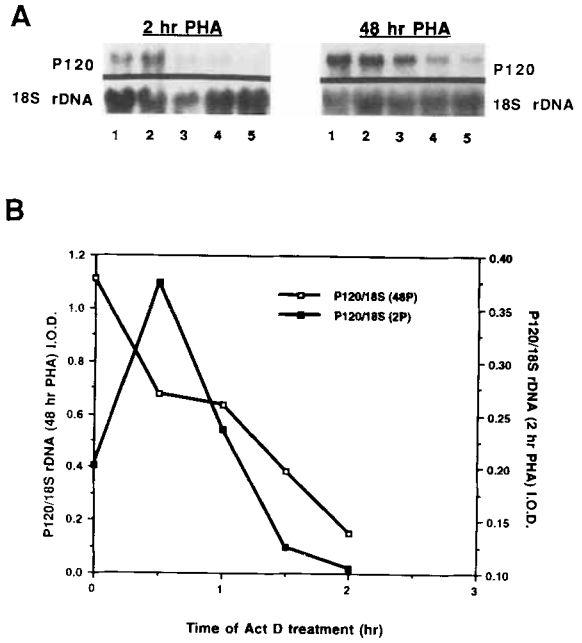

Fig. 3. Northern blot analysis of P120 mRNA stability in PHA-stimulated lymphocytes. Northern blots showing lymphocytes that were stimulated with PHA for 2 h or 48 h (**A**). PHA-stimulated lymphocytes are shown in *lane 1*. Cultures were treated with Act D (5 μg/ml) for 30 min (*lane 2*), 1 h (*lane 3*), 90 min (*lane 4*), or 2 h (*lane 5*). 30 μg of total RNA was loaded onto the gel and the blots were probed for P120 and 18S rRNA, as a loading control. **B** is a graphic representation of densitometric scans of the Northern blots in A.

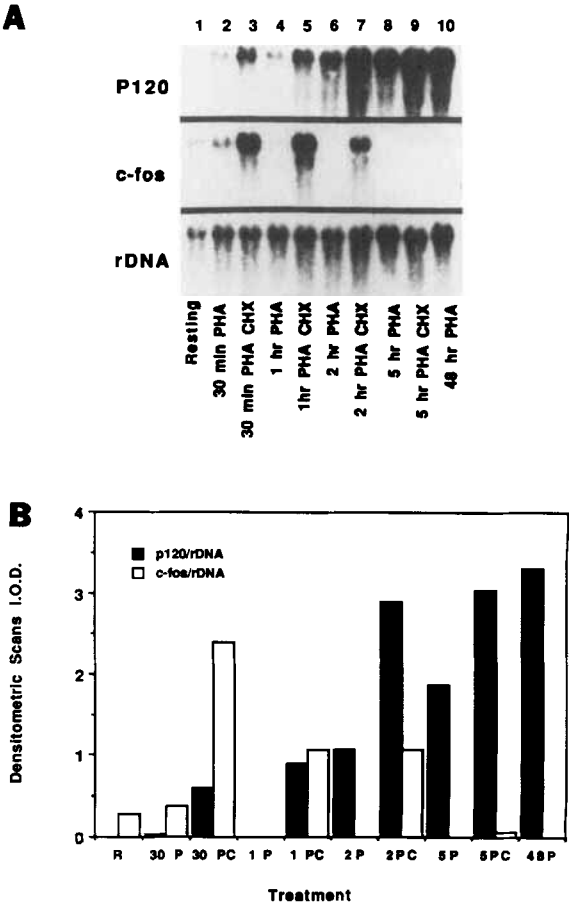

Fig. 4. Effect of protein synthesis inhibition on P120 mRNA accumulation in PHA-stimulated lymphocytes. Lymphocytes were plated at 3 × 10^6 cells/ml, stimulated with PHA (5 μg/ml) and treated with cycloheximide (CHX) (10 μg/ml) for various lengths of time. **A:** Northern blot analysis of P120 mRNA, *c-fos* mRNA, and 18S ribosomal RNA expression, resting lymphocytes (*lane 1*), 30 min PHA (*lane 2*), 30 min PHA and CHX (*lane 3*), 1 h PHA (*lane 4*), 1 h PHA and CHX (*lane 5*), 2 h PHA (*lane 6*), 2 h PHA and CHX (*lane 7*), 5 h PHA (*lane 8*), 5 h PHA and CHX (*lane 9*), 48 h PHA (*lane 10*). 30 mg of total RNA was loaded onto the gel. **B:** Densitometric scans of blots in A.

early gene, *c-fos*, p120 mRNA levels continue to increase as cells transverse G_1 towards S-phase (Table I, Fig. 4).

Inhibition of Protein Synthesis Increases the Half-Life of P120 mRNA and Increases the Rate of P120 Transcription

These findings suggest that cycloheximide may increase the transcription rate of P120 by inhibiting the expression of a specific repressor and/or may increase the stability of the p120 mRNA. These two possibilities were examined by assessing the stability of P120 mRNA and by determining the transcription rate of the P120 gene in resting and PHA-stimulated lymphocytes treated with cycloheximide, or with actinomycin D and cycloheximide. Figure 5 shows a representative Northern blot analysis of resting (panel A) and lymphocytes stimulated with PHA for 3 h (panel C). The blots were probed for P120 and 18S rRNA, as a loading control. It can be noted that by loading a large amount of total RNA (30 μg) and by long exposure of the autoradiograph, a low level of P120 mRNA could be detected in resting lymphocytes (Fig. 5A, lane 1). Lane 1 of each panel represents cells not treated with cycloheximide or actinomycin D. The resting or PHA-stimulated lymphocytes were treated with cycloheximide for 3 h (lane 2), or with actinomycin D for 1, 2, 3, or 4 h (lanes 3–6), or with both actinomycin D and cycloheximide for 1, 2, 3, or 4 h (lanes 7–10). Panels B and D show densitometric scans of the Northern blots in panels A and C, respectively. To control for unequal loading, the data points were reported as a ratio of O.D. readings between P120 and 18S rDNA.

Resting lymphocytes showed a trace amount of P120 mRNA (Fig. 5A, lane 1). However, rest-

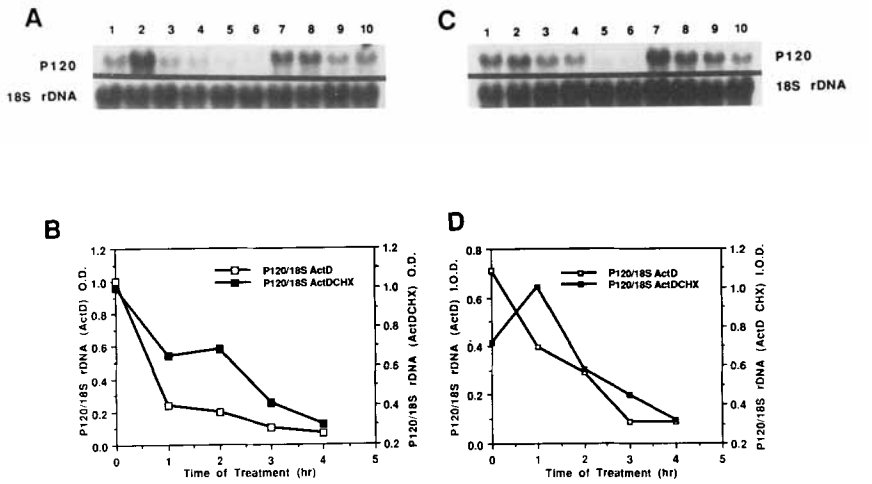

Fig. 5. Northern blot analysis of resting (**A**) and 3 h PHA-stimulated (**C**) lymphocytes. Control lymphocytes (*lane 1*) resting (A) or stimulated with PHA for 3 h (**B**). The resting lymphocytes or the PHA stimulated lymphocytes were treated with CHX only (10 μg/ml) for 3 h (*lane 2*); Act D only (5 μg/ml) for 1, 2, 3, and 4 h (*lanes 3–6*); or with CHX and Act D combined for 1, 2, 3, and 4 h (*lanes 7–10*). 30 mg of total RNA was loaded onto the gel. The same Northern blot was probed for P120, and 18S rRNA, as a loading control. B and **D** depict graphic representations of densitometric scans of the Northern blots in A and C, respectively.

ing lymphocytes treated with cycloheximide showed an increased accumulation of P120 mRNA (compare Fig. 5A, lane 1, with lane 2 of the same figure). This finding suggests that P120 mRNA expression is independent of protein synthesis. Futhermore, P120 mRNA was more stable in lymphocytes (resting or PHA-stimulated) treated with cycloheximide (Figures 5B and 5D). These results suggest that resting cells have a protein synthesis-dependent factor that prevents P120 mRNA accumulation in non-proliferating lymphocytes.

An increased transcription rate of P120 mRNA could also be responsible, at least in part, for the increased level of P120 mRNA observed in cycloheximide-treated cells. To ascertain whether this is true we determined the P120 transcription rate in lymphocytes treated with PHA, PHA and cycloheximide, or cycloheximide alone. The results are shown in Figure 6. The nuclear runoff assays (Fig. 6A) and the accompanying graphic depiction of densitometric scans of the blots (Fig. 6B) indicated that treatment with cycloheximide, with or without PHA, caused an increase in P120 transcription rate.

DISCUSSION

P120 is a growth-regulated [Freeman et al., 1989; Fonagy et al., 1992] nucleolar protein, the expression of which is required for G_1- to S-phase transition in lymphocytes [Fonagy et al., 1992]. P120 appears to be involved in ribosomal biogenesis and P120 expression correlates with the increased rRNA transcription during G_1 cell cycle progression [Wilson et al., 1993]. The role P120 plays in ribosomal biogenesis is presumptively through its putative role as a rRNA methyltransferase [Koonin, 1994]. The purpose of the present study was to determine how P120 is regulated as cells enter the cell cycle from a quiescent state. To accomplish this we studied the expression of P120 in mitogen-stimulated peripheral blood lymphocytes. Both P120 mRNA and P120 protein are undetected or present at very low levels in resting lymphocytes. P120 mRNA is expressed after *c-fos* mRNA in G_1-phase and prior to histone H3 mRNA expression.

This study indicates that P120 is regulated at both the transcriptional and post-transcriptional levels. Nuclear runoff experiments, measuring the transcription rate of P120, in resting and PHA-stimulated lymphocytes, indicate that as cells are stimulated to enter the cell cycle, the transcription rate of P120 increases. This suggests the presence of a transcriptional regulatory mechanism.

Other findings from this study also indicate that a post-transcriptional regulatory mechanism(s) is involved in the cell cycle related expression of P120. First, P120 mRNA accumulates in the presence of cycloheximide in PHA-stimulated and resting lymphocytes. Resting lymphocytes show very little P120 mRNA accumula-

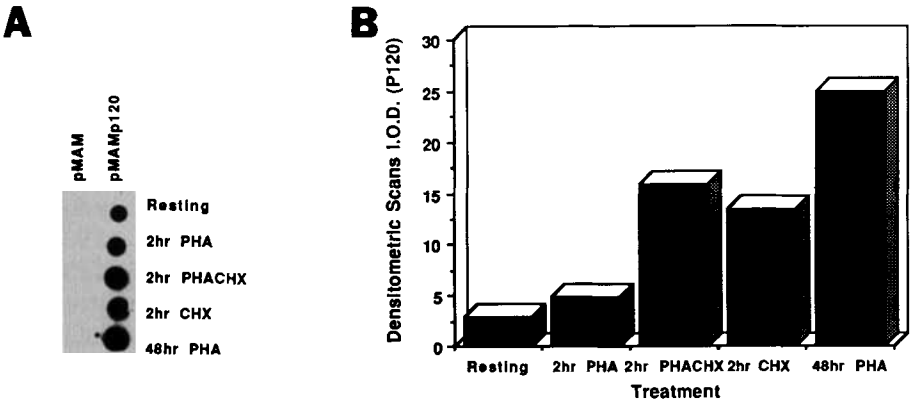

Fig. 6. Transcriptional rates for P120 using nuclear runoff analysis of lymphocytes treated with PHA (5 μg/ml), PHA and CHX (10 μg/ml), or CHX alone for various lengths of time. Following incubation, cells were harvested and their nuclei purified to be used to generate radiolabeled mRNA. 5 μg of the plasmids, pMAMp120 and pMAM, to control for non-specific binding, were hybridized onto the nitrocellulose prior to incubation with the radiolabeled RNA from the treated samples. **A:** Dot blot of nuclear runoff analysis. **B:** Densitometric scan of dot blots in A.

tion. Therefore, the increased amount of P120 mRNA observed in the presence of cycloheximide suggests that a protein synthesis-dependent factor(s) is present in resting lymphocytes; thus inhibiting the accumulation of P120 mRNA.

Second, nuclear runoff analyses showed that P120 is transcribed in resting lymphocytes, even though by Northern blot analysis very little P120 mRNA accumulation is observed. This suggests that the resting cell is poised to produce P120 protein by constantly transcribing the P120 gene and that the P120 transcript is rapidly turing over by a post-transcriptional regulatory mechanism. The plasmid used in nuclear runoff analysis of P120 transcription contained the entire P120 cDNA. Because of this, the possibility of premature termination of P120 transcription cannot by ruled out. Also, antisense transcription may have occurred off of the noncoding strand.

Finally, there is evidence that the stability of P120 mRNA is higher in S-phase lymphocytes than in early or mid-G_1 phase lymphocytes. Cycloheximide treatment also increases the stability of P120 mRNA in resting and PHA-stimulated lymphocytes. These findings imply that a post-transcriptional regulatory mechanism is also present in PHA-stimulated lymphocytes, and that this mechanism is at least partially sensitive to protein synthesis inhibition. These studies can not rule out the possibility that other post-transcriptional mechanisms exist at the protein level.

A transient increase in P120 mRNA was observed upon treatment with actinomycin D or cycloheximide. It is intriguing to note that in *E. coli* cells, the inhibition of protein synthesis stimulates rRNA synthesis [Nomura, 1987]. Treatment of bacterial cultures with chloramphenicol inhibits total cellular protein synthesis, but accelerates the synthesis of rRNA and tRNA. Chloramphenicol treatment has been shown to transiently stimulate stable RNA production and transcription of r-protein genetic units [Dennis, 1976]. It's possible that the inhibition of protein synthesis using cycloheximide in lymphocytes elicits a response from the mammalian cell similar to that seen in bacterial cultures. When protein synthesis is inhibited, the cell could react by increasing the production of rRNA, in order to bind up all of the available r-proteins to form intact ribosomes in an attempt to generate proteins.

As is true for other nucleolar proteins, such as nucleolin (C23) [Olson, 1983], and nucleophosmin (B23) [Feurstein and Mond, 1987], P120 protein expression is increased upon mitogenic stimulation of quiescent cells. However, C23 and B23 are expressed at much higher levels in the nucleolus in G_0 cells than is P120. C23 is thought to be multifunctional, possibly playing a role in pre-rRNA transcription and ribosome assembly [Bugler et al., 1982]. It is understandable that the levels of these proteins would be relatively low in or even absent from quiescent cells, because these cells have very inactive nucleoli, with very low ribosomal production rate [Busch and Smetana, 1970]. P120 protein probably functions in one of these nucleolar activities by virtue of its location alone, but the

fact that its regulatory patterns are similar to those of known nucleolar proteins also suggests a functional role in nucleolar activity. A role for P120 in ribosomal biogenesis is supported by a study that shows that antisense-mediated inhibition of P120 affects the accumulation of ribosomal precursor molecules [Wilson et al., 1993]. This data is supported by the recent finding that P120 may function as a rRNA methlytransferase [Koonin, 1994]. Other studies indicate that rRNA methylation may be required for proper processing [Cunningham et al., 1991] and assembly of preribosomes [Segal and Eichler, 1991].

P120 appears to be unique in the fact that it shares characteristics of both early G_1 gene products and late G_1- or S-phase proteins. The expression of P120 mRNA is independent of protein synthesis, a hallmark characteristic of immediate early genes. This is similar to that observed for the *c-fos* [Greenberg and Ziff, 1984], and *c-myc* [Campsi et al., 1984] proto-oncogenes. However, *c-fos* mRNA is expressed much earlier in G_1-phase than P120 mRNA and disappears rapidly. The expression of P120 mRNA is more similar to *c-myc* mRNA in timing of expression in mid- to late-G_1, but P120 persists for a longer period of time. P120 mRNA expression is also similar to the late gene product, thymidine kinase, which is expressed in late-G_1 and peaks at S-phase [Knight et al., 1989], but unlike P120 is sensitive to protein synthesis inhibition [Coppock and Pardee, 1985].

P120 also shares characteristics with another late gene product, proliferating cell nuclear antigen (PCNA), which is also growth regulated and is expressed primarily in late-G_1/S phase [Baserga, 1991], but P120 is expressed earlier in G_1 than is PCNA. Interestingly, evidence suggests that the PCNA gene, like the P120 gene, is actively transcribed in quiescent cells. Although both PCNA and P120 genes are transcribed in quiescent cells, little or no mRNA is detectable. However, PCNA differs from P120 in that the transcription rate of PCNA does not increase upon stimulation of the cells to proliferate [Almendral et al., 1988; Jaskuski et al., 1988]. It appears that the increase in accumulation of PCNA mRNA observed upon entry into the cell cycle is due to increased mRNA stability, similar to the increase in P120 mRNA stability as cells approach S-phase.

As is true for P120, evidence indicates that the cell cycle related expression of histone H3 is regulated at both the transcriptional and post-transcriptional levels [Alterman et al., 1984; Morris et al., 1991]. Alterman et al. [1984] showed that the transcriptional rate of histone H3 increased as the cells approached S-phase, but not enough to account for the large increase in histone mRNA observed prior to S-phase. Studies by Morris et al. [1991] suggested that the stability of histone H3 mRNA changes throughout the cell cycle. It appears from these studies that the regulation of histone H3 expression is tightly regulated throughout the cell cycle. Histone plays such a critical role in DNA synthesis, it is vitally important that the protein be present when needed, hence the need for such stringent control of expression.

Based on these results, we propose a possible model for the cell cycle related expression of P120 in Figure 7. In resting or quiescent cells a repressor molecule is present that suppresses the transcription of the P120 gene; some minimal transcription occurs. Also present in resting cells is a protein that degrades what little P120 mRNA is produced; therefore, little P120 mRNA accumulation is observed. Upon stimulation of lymphocytes by PHA (or treatment with cyclo-

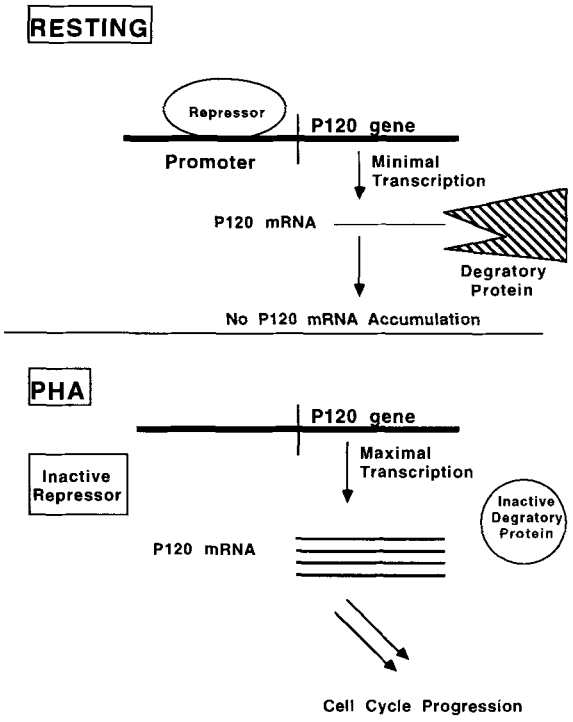

Fig. 7. Model depicting the possible regulatory mechanisms involved in the cell cycle related expression of P120 in resting and PHA stimulated lymphocytes.

heximide), the repressor becomes inactivated or is no longer present and the transcription rate of P120 is increased. In addition, the degratory protein becomes inactivated, or no longer produced, and the result is accumulation of P120 mRNA. Therefore, upon PHA stimulation the expression of P120 increases, and the cells can then progress through the cell cycle. This idea is supported by the antisense study of Fonagy et al. [1992] that indicated the expression of P120 protein is necessary for entry into S-phase.

It is understandable that a protein could be involved in both cell cycle progression and ribosomal biogenesis. Previous studies have shown that ribosomal biogenesis is closely tied to cell growth and, therefore, cell cycle progression [Freeman and Busch, 1991; Grummt, 1981]. The P120 protein could be a link between these two vital aspects of cell growth and function.

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