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NUCLEAR MATRIX PROTEIN MITOTIN messenger RNA IS EXPRESSED AT CONSTANT LEVELS DURING THE CELL CYCLE

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During the course of an investigation on nuclear matrix protein cDNAs, we have isolated a cDNA clone hybridizing with the messenger RNA encoding mitotin. Mitotin is a 125 kDa/pI 6.5 nuclear matrix protein present in proliferating but not in resting cells. This protein was shown to have a marked increase and characteristic redistribution in G2/M phase of the cell cycle. In this report, using synchronized Raji and WISH cells, we demonstrate that mitotin messenger RNA is expressed at the same level throughout the cell cycle.

Within the nucleus, there exist a highly structured framework which is termed nuclear matrix [1,2], and operationally defined as the residual structure after sequential extraction of nuclei with non-ionic detergents, nucleases and high-salt buffers [3]. Although the nuclear matrix is involved in many nuclear activities, including DNA replication, hnRNA processing and regulation of transcription [1,4,5,6,7], up to now other than lamins no nuclear matrix proteins have been cloned and characterized [8,9,10]. To palliate to this lack of information, we have constructed a small cDNA library using polysomal messenger RNAs immunoprecipitated with an antiserum against nuclear matrix proteins. One of the cDNAs was found to hybridize specifically to messenger RNA containing coding sequences for mitotin, a nuclear matrix protein expressed in proliferating cells [11,12].

Mitotin is a 125 kDa/pI 6.5 protein associated with the internal nuclear matrix [11]. This protein displays a speckled nucleoplasmic distribution throughout interphase and is markedly increased during late G2 and M phases of the cell cycle [12]. In the present study, we present evidence for the detection of mitotin messenger RNA and an analysis of the expression of this messenger RNA during the cell cycle.

MATERIALS AND METHODS

Cell Cultures. Human lymphoblastoid Raji cells (ATCC CCL86) were grown in suspension in RPMI 1640 medium (GIBCO) supplemented with 8% fetal calf serum.

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Human amniotic WISH cells (ATCC CCL25) were grown on MEM containing 5% fetal calf serum. Cells were synchronized by a double thymidine block [13].

Polyadenylated RNA preparation. A fraction rich in polysomes was prepared by the method of Ramsey and Steele [14] except that 1% Nonidet P-40 and 0.5% sodium deoxycholate were added during homogenization. The polysome pellet was resuspended in 10 mM Tris-HCl pH 7.6, 20 mM KCl, 2 mM magnesium acetate, and 20% (vol/vol) glycerol, at 200 A_{260} U/ml. After digestion with proteinase K, polyadenylated RNA was purified by two passages through oligo (dT)-cellulose.

Hybrid selection. Plasmid DNA was isolated from the clone 6A9 and attached to nitrocellulose filters. The filters were hybridized with 200 μg/ml polyadenylated RNA in a solution containing 65% formamide, 20 mM PIPES pH 6.5, 0.4 M NaCl, 0.2% sodium dodecylsulphate and 100 μg/ml yeast tRNA. Hybridization was performed at 50°C for 5 h. After intensive washing at 65°C, first with 10 mM Tris pH 7.6, 0.15 m NaCl, 1 mM EDTA, 0.5% SDS, followed by 0.2 x SSC, 0.2% SDS and finally 0.2 x SSC. The hybridized RNAs were eluted by boiling the filters in water containing 100 μg/ml tRNA and ethanol precipitated [15].

In vitro translation. Protein synthesis was carried out at 29°C for 60 min [16] in a solution containing the following components: 100 μl of nuclease-treated rabbit reticulocytes lysate, 10 mM HEPES pH 7.5, 0.2 mM GTP, 1 mM ATP, 2.5 mg/ml creatine phosphate, 0.5 mg/ml phosphocreatine kinase, 20 μM amino acids in a mix minus methionine, 1 mCi/ml [^{35}S]methionine (Amersham), 10 μg/ml hemin, 2.6 A_{260} U/ml yeast t-RNA, 0.5 mM spermidine, 1.5 mM magnesium acetate, 80 mM KCl, and hybrid-selected RNA.

Immunoprecipitation. For immunoprecipitation, translation products were incubated overnight at 4°C with 50 μg/ml of purified monoclonal anti-mitotin antibody [11], followed by addition of a suspension of IgG-Sorb (The Enzyme Center Inc.), and further incubation for 1 h at 22°C and 1 h at 4°C. The IgG-Sorb was then washed several times with lysis buffer [17], containing 0.5 M NaCl, followed by lysis buffer and finally by TBS (10 mM Tris-HCl pH 7.6, 0.15 mM NaCl, 1 mM PMSF). The bound proteins were eluted by boiling the IgG-Sorb in acrylamide-SDS gel electrophoresis sample buffer [18].

Protein electrophoresis. Electrophoresis in 8% polyacrylamide SDS gel was carried out as described [18]. The gels were treated with En^{3}Hance (New England Nuclear), dried, and exposed to radiographic films (Kodak RP-X-Omat). Two-dimensional electrophoresis was carried out according to O'Farrell [19].

Northern blot analysis. RNA isolation and analysis. Cytoplasmic RNA from Raji and WISH cells was isolated according to Sambrook *et al.* [20] as follows: the cells were lysed in 10 volumes of 10 mM Tris-HCl pH 7.9, 3 mM $CaCl_2$, 150 mM NaCl, 0.2 mg/ml heparin, 0.3% Triton X-100. After centrifugation at 15 000 x g for 10 min, the cytoplasm was treated with proteinase K and extracted with phenol-chloroform, RNA was separated in denaturating formaldehyde-agarose gels and transferred on Nytran (Schleicher and Schull) nylon membranes. Detection of specific RNAs was performed with a hybridization (according to the instruction of the supplier) at stringent conditions using a nick-translated 267 bp PstI/SphI fragment from the 5' end of the clone 6A9.

RESULTS AND DISCUSSION

To isolate cDNA clones coding for nuclear matrix proteins, polysomes were isolated from human lymphoblastoid Raji cells, and immunoprecipitated with an antiserum against BHK-21 nuclear matrix proteins, as described by [21,22]. This antiserum has previously been shown to label the nuclear envelope and an intranuclear structure in BHK cells [17]. Polyadenylated RNA was extracted from these immunopurified polysomes and used to constructed a small cDNA library.

The resulting library was screened using hybrid selection of polyadenylated RNA with the various cDNA clones, followed by *in vitro* translation of the hybrid selected RNA and characterization of the translation products by migration in SDS-PAGE. One of the clones, 6A9, was shown to recognize messenger RNA coding for a protein migrating at 125 kDa on SDS-PAGE (Fig. 1A) that was immunoprecipitated by the antiserum against BHK-21 nuclear matrix proteins used to construct the library (Fig. 1B).

Characterization of the translation product

Among the nuclear matrix proteins characterized, a nuclear matrix protein called mitotin had been described as migrating in the 125 kDa molecular weight region [11,12]. We decided to investigate if the translation product described in the previous section could correspond to mitotin. We first examined if the translation product was recognized by an anti-mitotin monoclonal antibody [11]. As in the previous experiment, polyadenylated RNA from Raji cells was hybrid-selected using the 6A9 clone and the hybrid-selected RNA was translated *in vitro* in rabbit reticulocyte lysate containing [^{35}S]methionine. The translation products were then incubated with the anti-mitotin monoclonal antibody and the immunoprecipitated polypeptides were analyzed by gel electrophoresis. As seen in Fig. 2A, the 125 kDa translation product was immunoprecipitated with the anti-mitotin monoclonal antibody. No band was observed when pGEM1 vector was used for the same experiment (Fig. 2B).

To confirm the identity of the translation product as mitotin, immunoprecipitates of *in vitro*-translated products were analyzed on a two-dimensional gel. In parallel, nuclear matrix proteins from Raji cells were separated [11], transferred on nitrocellulose membrane and subjected to a immune reaction with the anti-mitotin antibody and peroxidase conjugated anti-mouse immunoglobulins. The [^{35}S]methionine-protein comigrated with mitotin identified on the gel ran in parallel and immunoblotted (Fig. 3A

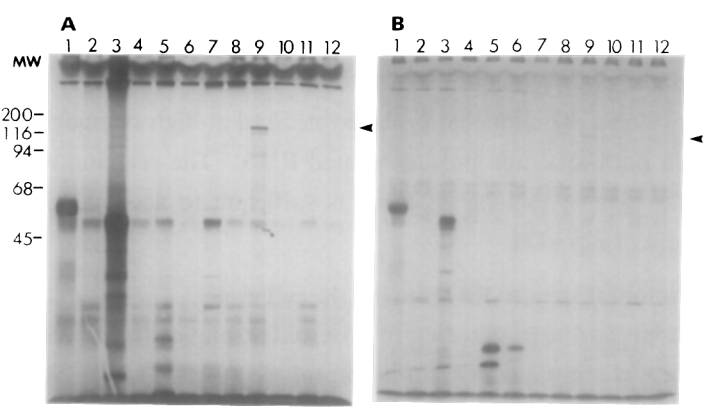

Figure 1. *In vitro* **translated products from polyadenylated RNA hybrid-selected with cDNA clones from the 6A series.** **A-** Fluorogram from a SDS-PAGE of the *In vitro* translated products. **B-** Fluorogram from a SDS-PAGE of the *in vitro* translated products immunoprecipitated with an antiserum against BHK-21 nuclear matrix proteins. An arrow indicates the presence of a 125 kDa protein.

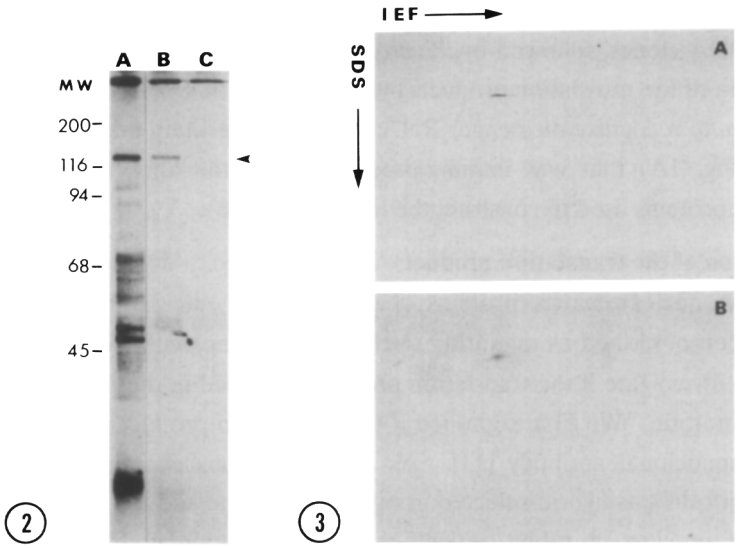

Figure 2. **Immunoprecipitation with an anti-mitotin antibody.** Polyadenylated RNA was hybrid-selected with either the 6A9 cDNA clone (**A, B**) or the pGEM1 vector (**C**). *In vitro* translated products were immunoprecipitated (**B, C**) and analyzed by SDS-PAGE and autoradiography. An arrow indicates the presence of the 125 kDa protein.

Figure 3. **Two-dimensional polyacrylamide gel analysis. A-** *In vitro* translated product of polyadenylated RNA hybrid-selected with the 6A9 clone and immunoprecipitated with the anti-mitotin monoclonal antibody. **B-** Raji cell nuclear matrix proteins reacted with the anti-mitotin monoclonal antibody.

and 3B). These experiments established that the messenger RNA hybridizing with the 6A9 clone encoded mitotin.

Size determination of mitotin messenger RNA

To characterize the messenger RNA hybridizing with the 6A9 clone, total, polyadenylated and poly(A^-)RNA (Fig. 4) from Raji cells were fractionated by electrophoresis in a denaturating formaldehyde/agarose gel and transferred onto a nylon membrane. These RNAs were hybridized with a nick-translated 267 bp-fragment from the 5' end of the 6A9 clone. The labeled probe hybridized at high stringency to a single band of 4.5 kilobases in both total and polyadenylated RNA. The reaction with poly(A^-)-RNA was negative. The size of this 4.5 kb RNA is sufficient to accommodate the sequences coding for a protein of 125 kDa.

Expression of mitotin messenger RNA during the cell cycle

Although mitotin has been detected in all proliferating cells, the amount of protein is about 7 fold higher in mitotic cells than in interphase cells [[11,12]. To determine if this drastic change was due to a increase in the amount of messenger RNA, we have analyzed the level of mitotin messenger RNA during the cell cycle. For this purpose Raji and WISH cells were synchronized with a double thymidine block [13]. Cells were collected at various times after release from the block, for Raji cells these times were 3, 7 and 12 h respectively

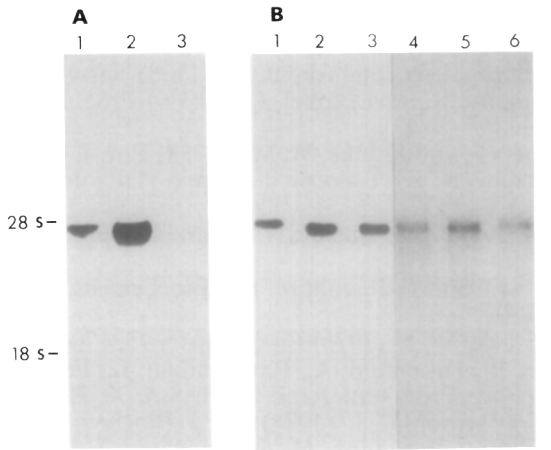

Figure 4. **Northern blot analysis of messenger RNA hybridizing with the 6A9 cDNA clone.** **A**- Total cytoplasmic RNA (1), polyadenylated RNA (2), or poly(A$^-$)RNA (3) from Raji cells was separated on denaturating 1% agarose-formaldehyde gels and hybridized with a nick-translated cDNA probe from the 6A9 clone. **B**- RNA from synchronized Raji (1, 2, 3) and WISH cells (4, 5, 6) hybridized with the nick-translated probe from clone 6A9. S phase:(1 and 4), G2 phase:(2 and 5), G1 phase:(3 and 6). Migration of the 28S and 18S RNA is indicated.

for the S, G2 and G1 phases, while for WISH cells the corresponding times were 3, 8 and 14 h. Total RNA from each cell cycle phase was extracted and fractionated by electrophoresis in a denaturating formaldehyde/agarose gel and transferred onto a nylon membrane. The RNAs were hybridized with the nick-translated 267 bp-probe described in the previous section. As visualized in fig. 4 the level of mitotin messenger RNA is nearly constant during the cell cycle with a slight increase (less than two fold) in G2 phase.

These results are in good agreement with the recent results for the synthesis and stability of mitotin [23]. The protein was shown to be synthesized throughout the whole cell cycle. The marked accumulation of mitotin in mitotic cells was found to be a result mainly of a phosphorylation stabilization taking place during the G2 phase of the cell cycle.

In conclusion, in the present study we have shown that the nuclear matrix protein mitotin is encoded by a 4.5 kb messenger RNA, and that although the protein level markedly varies, this mitotin messenger RNA is expressed at constant levels throughout the cell cycle .

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