2′,5′-Oligoadenylate-dependent RNAase located in nuclei: biochemical characterization and subcellular distribution of the nuclease in human and murine cells

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A cellular fractionation procedure allowed the rapid preparation of membraneless nuclei which contained a 2′,5′-oligoadenylate (2-5A)-binding activity which was not due to cytoplasmic contaminants. Purified nuclei prepared from human lymphocytic leukaemia cells and mouse fibroblasts were found to contain 20–22% of the total cellular enzyme. In contrast with the cytoplasmic enzyme which was only present in a 2-5A-free form, 75% of the 2-5A-binding activity was found in the nuclei after a denaturing–renaturing procedure as the 2-5A-binding site was masked. Although the purification of nuclei from mouse fibroblasts was less effective, it appeared that, in confluent and growing cells, 50% and 75% respectively of the 2-5A-binding site was masked. Additional findings obtained by partial proteolysis and two-dimensional gel analysis provided definitive data on the nuclear location of this enzyme. Study of the nuclear 2-5A-dependent RNAase with a 2-5A-masked site could lead to an understanding of the molecular pathway involved in single-stranded RNA stability.

INTRODUCTION

Interferons are synthesized and secreted in response to viral infection and other stimuli. Specific binding of interferon-α to cell surface receptors activates a tyrosine kinase, designated tyk 2 (Firmbach-Kraft et al., 1990; Fu, 1992; Velasquez et al., 1992), which phosphorylates the cytoplasmic multiprotein transcription factor interferon-stimulating gene factor-3 (ISGF-3) (Levy et al., 1989; Fu et al., 1990, 1992; Schindler et al., 1992) leading to its nuclear translocation, and transcription of a limited set of genes (Sen, 1991). It has been subsequently suggested that the cell-produced proteins are implicated in antiviral activities, as well as in the control of cell growth and differentiation (Stark et al., 1979; Johnston and Torrence, 1984).

One of these induced proteins is a 2-5A-dependent endo-RNAase [where 2-5A is a set of 2′,5′-linked adenylic oligoribonucleotides of the form $p_x(A2'p)_nA$ with $x = 2$ or 3 and $n > 2$] (Zhou et al., 1993). The 2-5A effectors regulate the cleavage of single-stranded RNA by activating this nuclease. Thus 2-5A-dependent RNAase is postulated to be in either an inactive form free of 2-5A or an active 2-5A–RNAase complex form having a masked 2-5A-binding site. The 78–80 kDa nuclease is found in very small quantities, and very sensitive assays are required for its detection. Levels of the 2-5A-dependent RNAase with an unmasked 2-5A-binding site can be directly detected in subcellular fractions by affinity-labelling methods using a high-specific-radioactivity (3×10^6 Ci/mol) derivative of 2-5A (Silverman et al., 1981). In contrast, enzyme populations with a masked 2-5A-binding site need to be denatured and renatured in order to remove 2-5A or other associated molecule before labelling with a 2-5A radiolabelled probe (Bayard and Zhou, 1992). This finding led us to investigate whether this enzyme is present in the nucleus of cultured human and murine cells. Such information might provide clues to the mechanisms involved in the controlled turnover of cellular mRNAs.

Controversial claims have been made about the location of 2-5A-dependent RNAase in the nucleus. The persistence of this doubt seems to us to result from the poor characterization of the isolated nuclei to date. In contrast with the well-established 78–80 kDa cytosolic enzyme (Wreschner et al., 1981a,b, 1982; Silverman et al., 1988; Nolan-Sorden et al., 1990), the putative homologous nuclear enzyme is biochemically ill defined. In 1981, Nilsen et al. suggested a possible role of the 2-5A pathway in nuclear RNA metabolism, and St. Laurent et al. (1983) detected several 2-5A-binding proteins in the nuclear wash fraction from Ehrlich ascites tumour cells. However, Salhezada et al. (1991), on the basis of immunofluorescence and biochemical studies, reported that 2-5A-dependent RNAase did not have a nuclear location. Therefore we approached this question more directly by using a fast and efficient procedure to purify nuclei. The results reported in this paper support the endogenous presence of the enzyme in the nucleus, and provide additional evidence delineating distinct characteristics of the nuclear and cytosolic forms of the enzyme.

EXPERIMENTAL

Materials

Media for cell cultures were obtained from GIBCO–Bethesda Research Laboratories, and sera were from Seromed. T_4 RNA ligase was from Pharmacia LKB Biotechnology Inc. [^{32}P]pCp (specific radioactivity, 110 TBq/mmol) and the enhanced chemiluminescence Western-blotting system was from Amersham Corp. Human interferon-α was a gift from Dr. Carcagne (Institut Mérieux, Lyon, France). Nitrocellulose paper (0.45 μm pore size) was from Schleicher and Schüll. SDS, 2-mercaptoethanol and all chemicals for electrophoresis were from Bio-Rad. Nuclei were treated with either protease-free DNAase I from GIBCO–BRL or in some cases with the DNAase I preparation from Boehringer, which has protease activity. Avidin–horseradish

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; HRP, horseradish peroxidase; [^{32}P]pCp, cytidine 3′,5′-[5′-^{32}P]bisphosphate; 2-5A, 2′,5′-oligoadenylate; core 2-5A, 5′-dephosphorylated 2-5A or $(A2'p)_nA$.

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peroxidase (HRP), Tween 40 and Tergitol NP14 were from Sigma. Proteins were estimated by using the ready-to-use Coomassie Blue G-250 reagent from Pierce. The transfer apparatus from Bio-Rad (Trans-blot cell model) was modified so that a distance of 4 cm was present between the two planar electrodes.

Buffers

Buffer A consisted of 10 mM Tris/HCl, pH 7.6, 2 mM $MgCl_2$, 75 mM NaCl, 1 mM phenylmethanesulphonyl fluoride (PMSF) and 2% (v/v) Tween 40; buffer B consisted of 10 mM Tris/HCl, pH 7.6, 75 mM KCl, 1 mM EDTA, 7 mM 2-mercaptoethanol and 1 mM PMSF; buffer C was 11 mM Hepes/KOH, pH 7.6, 104 mM KCl, 5.8 mM magnesium acetate, 8.8 mM 2-mercaptoethanol, 1.2 mM ATP, pH 7.6, 100 μg/ml leupeptin and 1 mM PMSF; buffer D was 50 mM Tris/HCl, pH 7.6, 150 mM 2-mercaptoethanol, 40 mM magnesium acetate, 5 mM EDTA and 2% (v/v) Tergitol NP14 or 0.1% Tween 20; buffer E was 20 mM Hepes/KOH, pH 8.2, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol and 25% (v/v) glycerol.

Preparation of cytosol extracts and isolated nuclei

Spleens, brains and kidneys (from 16-week-old male Swiss mice) were cut into small pieces at 4 °C and suspended in 2 vol. of lysis buffer A. Nuclei were obtained as described below. Murine L929 and human lymphocytic leukaemia cells (CEM and Jurkat) were grown and maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum. PBS-washed cells (1×10^7) were resuspended in 300 μl of buffer A. Cells were disrupted by 30 strokes in a tight-fitting Dounce homogenizer and the homogenate was centrifuged at 2000 *g* for 1 min. The supernatant or cytosolic extract was pipetted off and stored in liquid nitrogen. The pellet was resuspended in 300 μl of buffer B and layered on to 750 μl of 50% (w/v) sucrose in buffer A. The pellet of nuclei obtained after centrifugation for 2 min at 11000 *g* was resuspended in 300 μl of buffer B and stored in liquid nitrogen. Nuclei were stable to freezing and thawing when stored in liquid nitrogen: no breakdown of nuclei or loss of 2-5A-binding activity was observed after 3 months. For preparation of nuclear extracts, the nuclei were successively suspended in 300 μl of buffer E containing NaCl at concentrations ranging from 0.1 to 0.4 M. For each salt concentration the suspension was gently mixed for 15 min at 4 °C before centrifugation at 1000 *g* for 1 min at 4 °C. The supernatant was analysed by SDS/PAGE, and the pellet containing the nuclei was resuspended with a higher NaCl concentration in buffer E for another cycle.

Characterization of purified nuclei

The morphology and apparent structural integrity of the nuclei were assessed by electron microscopy. Pellets of nuclei isolated from the three cell lines mentioned above were fixed with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5, then postfixed with 1% (w/v) OsO_4 buffered with 50 mM veronal/acetate/HCl, pH 7.5, for 1 h at 4 °C. Samples were then embedded in Epon, using conventional methods for electron microscopy. After sectioning and contrast staining with uranyl lead, nuclei were observed and microphotographs were taken with a JEOL 1200 electron microscope operating under a 70 kV accelerating voltage.

The presence of biotin-dependent carboxylases in the cytoplasm and nuclei was assessed as follows: proteins from cytosolic extract and DNAase I-treated nuclei were denatured in 5% (w/v) SDS, subjected to SDS/PAGE (10% gels) and electrotransferred on to nitrocellulose. After renaturation of proteins as previously described (Bayard and Zhou, 1992), biotin-containing carboxylases were detected by incubation with avidin–HRP for 1 h and revealed by using the enhanced chemiluminescence Western-blotting system. Enhanced chemiluminescence was detected with an X-Omat RP film for 10 s.

Detection and estimation of 2-5A-binding proteins

Synthesis and purification of $p(A2'p)_2(Br^8A2'p)_2A3'$-[^{32}P]Cp

The bromine-substituted 2-5A derivative $p(A2'p)_2(Br^8A2'p)_2A$, (a gift from Dr. P. Torrence) was chemically synthesized and covalently labelled at its 3'-OH end by linkage of the donor [5'-^{32}P]pCp (specific radioactivity 3×10^6 Ci/mol) with the T_4 RNA ligase as previously described (Silverman et al., 1981). After ligation, the product was purified by h.p.l.c. by the method of Brown et al. (1981).

Measurement of 2-5A-binding activity by the non-covalent radiobinding assay

The affinity of $p(A2'p)_2(Br^8A2'p)_2A3'$-[^{32}P]Cp for 2-5A-dependent RNAase was determined in a radiobinding assay as previously described (Silverman et al., 1981; Silverman and Krause, 1987). The assays were performed in a final volume of 50 μl of buffer C containing the radiolabelled probe (3 fmol, about 20000 c.p.m.) and 140 μg of protein. Incubation mixtures were placed on ice for 15 h after which the reaction mixtures were spotted on to 1 cm^2 pieces of dry nitrocellulose. The filters were washed twice by swirling in cold distilled water for 5 min in order to remove the unbound probe. The levels of radioactivity retained on the nitrocellulose were determined by scintillation counting of the dried filters.

Photoaffinity covalent labelling of 2-5A-dependent RNAase

Cytosolic extract and nuclei (140 μg of protein) were incubated with 100 units of DNAase I and 100000 c.p.m. of bromine-substituted probe in buffer C for 15 h at 4 °C before being cross-linked under a u.v. lamp (310 nm, 15 W) for 3 h at 4 °C (Nolan-Sorden et al., 1990). The irradiated mixture was incubated with SDS sample buffer and analysed for radiolabelled 2-5A-binding protein(s) by SDS/PAGE (10% gels) or two-dimensional gel electrophoresis as described below. The gel was dried and exposed with an X-Omat RP film at −80 °C for 4 h.

Denaturing–renaturing affinity-blotting assay

The detection of 2-5A-binding protein(s) is affected by the presence of endogenous 2-5A or 2-5A derivative(s) which compete with the probe. We used the procedure of affinity-blotting based on denaturing–renaturing conditions allowing the removal of bound and free 2-5A from 2-5A-binding protein(s) to assess total enzyme in different cellular fractions (Bayard and Zhou, 1992). Briefly, cytosolic extract and nuclei containing 140 μg of proteins were digested with 100 units of DNAase I for 15 h at 4 °C. The mixture was denatured with 5% (w/v) SDS sample buffer at 100 °C for 3 min and proteins were separated by SDS/PAGE (10% gels). After migration, the slab gel was electroblotted at 4 °C (550 mA for 2 h) on to nitrocellulose (0.45 μm pore size) essentially as described by Burnette (1981). The nitrocellulose sheet was then blocked for 1 h at 20 °C with 2% (w/v) skimmed milk in buffer D. Renaturation of the enzyme was accomplished by incubating the blots at 4 °C for 30 h in buffer D containing the probe $p(A2'p)_2(Br^8A2'p)_2A3'$-

[^{32}P]Cp (60000 c.p.m./ml). After concomitant renaturation and binding of the radiolabelled probe, the nitrocellulose was washed in cold distilled water for 5 min to remove non-specific binding and air dried. The affinity-labelled 2-5A-dependent protein(s) or polypeptides were located after autoradiography with a Kodak X-Omat RP film at −80 °C for 4 h.

Two-dimensional gel electrophoresis

Proteins (140 μg) from cytosolic extract and nuclei were prepared as described above using the photoaffinity-labelling procedure. 2-5A affinity-labelled proteins were analysed by two-dimensional non-equilibrium pH-gradient electrophoresis (O'Farrell et al., 1977). Proteins were separated in one-dimensional cylindrical gels with a 1.2% (v/v) ampholine pH range 2.5–4.5 in the presence of 2% (v/v) Nonidet P40. The gels were run at 400 V for 2 h. The proteins were then transferred to 10% polyacrylamide gels. The pH gradient after isoelectric focusing was determined by slicing the gel into 3 mm sections, soaking each in distilled water for 15 min and reading the pH of the water on a pH-meter. The two-dimensional slab gels were dried and exposed to a Kodak X-Omat RP film at −80 °C for 15 h.

RESULTS

Purification of nuclei

In order to describe nuclear proteins it is essential to purify nuclei so that they are free of cytoplasmic contaminants. Therefore cells were ruptured by homogenization in buffer A containing 2% (v/v) Tween 40 in order to solubilize the membranes. As assessed by electron microscopy, the nuclear envelope was removed by the detergent, and intact nuclei were obtained by centrifugation through a cushion of sucrose. Nuclei prepared from CEM and Jurkat cells were totally free from cytoplasm. When isolated from L929 cells, membrane removal was complete but remnants of non-membranous organelles, such as ribosomes and intermediate filaments, were found around the nuclei (results not shown).

Contamination of nuclear preparations with cytoplasmic components was assessed by determining the levels of biotin-dependent carboxylase, as this enzyme is only located in the cytoplasm (O'Leary, 1992). As shown in Figure 1, analysis with avidin–HRP allowed the detection in the cytoplasm of two (78 and 80 kDa) carboxylases in human CEM and Jurkat cell lines, and an additional 120 kDa form was detected in murine L929 fibroblasts. Only 2–3% of the 120, 78 and 80 kDa forms were recovered in nuclei of L929 cells and only trace amounts were seen in nuclei of CEM and Jurkat cells.

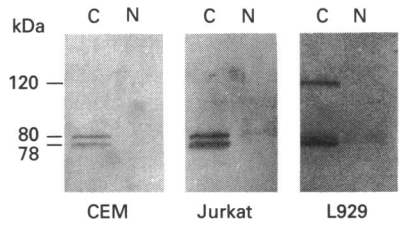

Figure 1 Chemiluminescence detection of biotin-dependent carboxylases in nuclei (N) and cytoplasm (C) of mouse L929 and human CEM and Jurkat cell lines

Proteins (140 μg) were denatured in SDS, subjected to SDS/PAGE (10% gels) and electroblotted on to nitrocellulose. Renaturation was achieved by incubating the blot in buffer D for 30 h at 4 °C. Biotin-dependent carboxylases were revealed with avidin–HRP hybridization and an enhanced chemiluminescence Western-blotting system (Amersham). The film was exposed for 10 s.

Leakage from the nuclei is a more difficult problem when studying levels of low-molecular-mass proteins, as nuclear constituents may be lost by passive diffusion during purification of the nuclei, as previously described for nucleoplasmin (Laskey and Earnshow, 1980). The present rapid isolation of nuclei, taking less than 4 min, minimized passive diffusion of 2-5A-dependent RNAase from the nuclei. This was investigated with a preparation of human kidney, in which 2-5A-dependent RNAase was detected only in the nuclei and was completely absent from the cytoplasm, confirming that no diffusion had occurred during the preparation of the nuclei (B. Bayard, unpublished work). In addition, no leakage of 2-5A-dependent RNAase from purified nuclei was observed even after storage in buffer B for 3 months in liquid nitrogen. It seems likely that there are no losses of the enzyme by diffusion from the nuclei during the fractionation and storage procedure.

2-5A-dependent RNAase levels in human CEM and Jurkat cell lines

As 2-5A-dependent RNAase may exist in a 2-5A-free form (open or unmasked binding site) or complexed with 2-5A (closed or masked binding site) in cytoplasm and nuclei, three assays for this enzyme have been used in this study. Non-covalent radiobinding and u.v. photo-cross-linking methods have proved to be particularly useful in measurement of the level of 2-5A-free nuclease in cell extracts. Measuring the total enzyme in a cell extract required removal of endogenous and bound 2-5A which could compete with the probe. A denaturing–renaturing affinity-blot assay (Bayard and Zhou, 1992) was thus used to assess total RNAase in different cellular fractions. For all these assays, the binding was specific for 2-5A, as it was abolished by addition of unlabelled 2-5A to the assay mixtures (results not shown).

Only traces of an 80 kDa 2-5A-binding protein were detected in purified nuclei from CEM and Jurkat cells as compared with higher levels found in the cytoplasm and estimated to be 4.7–6.5 fmol per mg of protein. When endogenous or bound 2-5A was removed from the enzyme by denaturation–renaturation, the level of total enzyme present in the cytoplasm of CEM and Jurkat cells was unchanged whereas it significantly increased in the nuclei (Figure 2). It represented around 80–90% of the cytoplasmic form or 20–22% of the total. The 2-5A-free form of the enzyme was mainly present in the cytoplasm and the 2-5A–RNAase complex was located in the nuclei. This makes the cytosolic form distinct from its homologous nuclear counterpart. This distribution is not restricted to CEM and Jurkat cells, but appears to be the same in extracts from lymphoblastoid Daudi cells (results not shown). In addition, levels of the different forms of 2-5A-dependent RNAase were found to be unchanged after treatment with human α,β-interferon (500 units/ml) (results not shown).

2-5A-dependent RNAase levels in murine L929 cells

Jacobsen et al. (1983) reported that the level of this enzyme increased in JLS-V9R monolayer culture during the transition from the subconfluent growing state to the confluent stationary phase. It was therefore of interest to determine whether the levels of the two forms of 2-5A-dependent RNAase fluctuate as a function of the growing or confluent stationary state in both nuclei and cytosol of L929 cells.

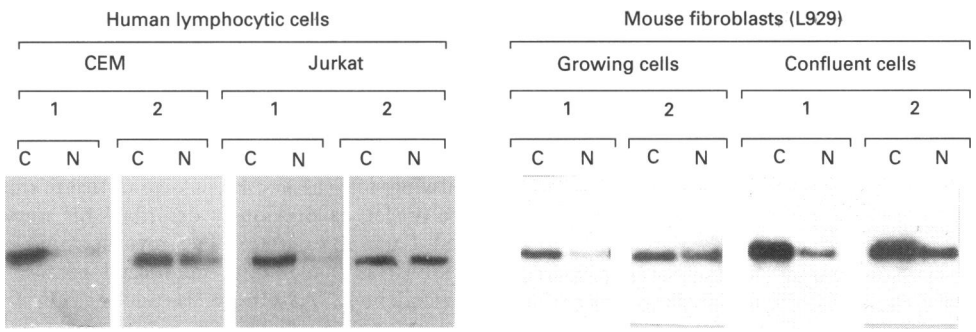

Figure 2 Levels of 2-5A-free (1) and total (2) 2-5A-dependent RNAase in cytosol (C) and purified nuclei (N) prepared from human lymphocytic cells (CEM and Jurkat) and mouse fibroblasts

The 2-5A-free form of the enzyme was estimated by the u.v. cross-linking procedure and total 2-5A-dependent RNAase was measured by the denaturing–renaturing affinity-blot assay. Each lane contained 140 μg of protein.

Table 1 Relative amounts of total 2-5A-dependent RNAase extracted from nuclei of CEM, Jurkat and L929 cells by increased NaCl concentrations

Nuclei were successively suspended for 15 min at 4 °C in buffer E containing NaCl concentrations ranging from 100 to 400 mM. The relative levels of 2-5A-dependent RNAase were obtained by the denaturing–renaturing affinity-blot assay. The data from each cell line have been normalized.

		2-5A-dependent RNAase (%)			
Cell lines	NaCl concentrations (mM)...	100	200	300	400
CEM		0.5	99.5	0	0
Jurkat		0.5	36.1	40.3	23.1
L929		58.8	31.9	7.8	1.5

In growing cells (measured 24 h after subculturing of a confluent cell layer), the levels of the 2-5A-free form of the enzyme were 19.9 and 6.6 fmol/mg of protein in the cytosolic extract and nuclei respectively, as determined by the radiobinding assay or the u.v. photo-cross-linking procedure (Figure 2). As the cells became confluent, the levels of the 2-5A-free form increased 2-fold in both the cytoplasm and nuclei (49.3 and 13.6 fmol/mg of protein). Estimation of total 2-5A-dependent RNAase in the cytosolic extract showed no variation between confluent and growing cells, confirming that the enzyme was mainly present in a 2-5A-free form (open binding site in this location). More significant differences between the levels of total and 2-5A-free enzyme were found in the nuclei (Figure 2), demonstrating that 2-5A-dependent RNAase was in a 2-5A–RNAase complex in 50 and 70 % of nuclei of confluent and growing cells respectively. Therefore it is likely that the enzyme in the nuclei mainly had a 2-5A-bound or -masked site.

Optimal NaCl concentration for 2-5A-dependent RNAase extraction from purified nuclei

To achieve selective removal of 2-5A-dependent RNAase from nuclei, we examined several NaCl concentrations for its extraction and diffusion efficiency. The optimum NaCl concentration was determined by successively suspending purified nuclei for 15 min at 4 °C in buffer E containing NaCl concentrations ranging from 0.1 to 0.4 M. Small amounts of the enzyme can be detected in the nuclear wash with the sensitive u.v. cross-linking method, which measures only the 2-5A-free form. Therefore the level of the enzyme in the nuclear wash was assessed by the denaturing–renaturing affinity-blot method, allowing quantification of both the 2-5A-free and -bound forms. As shown in Table 1, differences in the diffusion of the enzyme from the nuclei were observed between these three cell lines, mainly between human and mouse. Mouse L929 nuclear wash prepared with 100 mM NaCl contained about 59 % of the nuclease, whereas only traces were detected in the human CEM and Jurkat counterparts. For CEM cells, 2-5A-dependent RNAase was removed from the nuclei with 200 mM NaCl, whereas the enzyme diffused from Jurkat-cell nuclei at 200, 300 and 400 mM NaCl.

Proteolytic cleavage of cytoplasmic and nuclear 2-5A-dependent RNAase

To establish possible structural differences between 2-5A-dependent RNAase from nuclei and cytosol of L929, CEM and Jurkat cell lines, limited enzymic degradation was performed with papain, trypsin, endoproteinase Glu-C (V_8) and an unknown protease present in the commercial pancreatic DNAase I preparation from Boehringer. The fragments containing the 2-5A-binding domain were u.v. photo-cross-linked with $p(A2'p)_2(Br^8A2'p)_2A3'$-[^{32}P]Cp for 3 h at 4 °C, and products were analysed by SDS/PAGE. Limited digestion of the nuclease with papain, trypsin and V_8 led to a pattern of peptide fragments that was specific for each protease used but identical for the cytoplasmic and nuclear forms of the enzyme (results not shown). When proteolysis was performed with the DNAase I preparation contaminated with unknown protease(s), specific cleavages were observed for the enzymes of cytoplasmic and nuclear origin. This is particularly clear-cut for murine L929 fibroblasts (Figure 3, lane 3). The nuclear form was cleaved into a main fragment of 36 kDa, whereas the cytoplasmic form was more resistant, producing a polypeptide of 40 kDa plus other peptides. A similar pattern of degradation was obtained for 2-5A-dependent RNAase from the cytosol and nuclei of mouse spleen, brain and kidney (Figure 3, lanes 4, 5 and 6). Traces of a 40 kDa polypeptide observed in the nuclear fractions of L929 cells as well as in murine tissues might be due to the presence of cytoplasmic contaminants. Less clear cleavage differences were obtained for human CEM and Jurkat cell 2-5A-dependent RNAase (Figure 3, lanes 1 and 2). Two main cleavage products of 36 kDa and 18 kDa were detected in both cytosolic extract and nuclei.

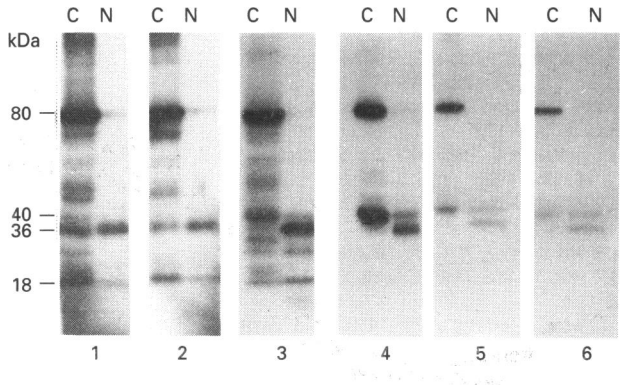

Figure 3 Proteolytic cleavage of u.v. cross-linked 2-5A-dependent RNAase from cytosolic (C) and nuclear (N) preparations of CEM (1), Jurkat (2), L929 (3) cells and mouse spleen (4), brain (5) and kidney (6)

Proteins (140 μg) from cytosol and nuclei were incubated with both pancreatic DNAase I with proteolytic activity from Boehringer (100 units, grade I) and the bromine-substituted analogue of 2-5A. The probe was cross-linked to 2-5A-dependent RNAase, and radiolabelled polypeptides were obtained on SDS/PAGE. The sizes of the 2-5A-dependent RNAase and major polypeptides are indicated on the left.

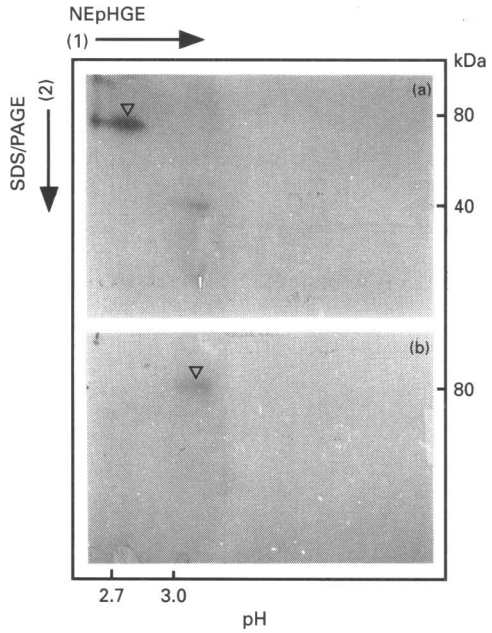

Figure 4 Two-dimensional gel electrophoresis of 2-5A-binding proteins from murine confluent L929 cells

Proteins (140 μg) from cytosolic extracts (**a**) and purified nuclei (**b**) were covalently labelled with the probe p(A2′p)$_2$(Br^{8}A2′p)$_2$A3′-[^{32}P]Cp and electrophoretically resolved in two-dimensional gels. The first dimension was non-equilibrium pH-gradient electrophoresis (NEpHGE) with a pH scale of 2.5–4.5. The second dimension was SDS/PAGE (10% gels). Labelled proteins were visualized by autoradiography with a Kodak X-Omat-RP film for 15 h. The molecular-mass range of the second-dimension gel is shown on the right. The pI values of the 80 kDa 2-5A-labelled enzyme from cytosolic extract (pI 2.7) and nuclei (pI 3.0) are indicated below.

Two-dimensional gel analysis

To reveal differences in charge between cytosolic and nuclear 2-5A-dependent RNAase, 140 μg of proteins from each fraction were subjected to two-dimensional gel electrophoresis (non-equilibrium pH-gradient electrophoresis and SDS/PAGE). By analogy with the denaturing–renaturing affinity-blot assay, which estimates the 2-5A-binding proteins after SDS/PAGE, we tried to detect the enzyme immobilized on nitrocellulose after two-dimensional gel electrophoresis. We failed to renature the 2-5A-binding site of the enzyme after denaturation with urea/SDS and treatment with ampholines. As the enzyme from CEM- and Jurkat-cell nuclei was mainly in a 2-5A-masked conformation, we have not been able to detect it after two-dimensional gel electrophoresis. This is not the case for L929 confluent cells which contain a higher level of the 2-5A-free form in the nuclei. This form of the enzyme was therefore covalently labelled with the probe using the u.v. cross-linking method before its electrophoretic migration. As shown in Figure 4, the cytosolic 2-5A-dependent RNAase from L929 cells was resolved into a major 80 kDa spot with an isoelectric pH of 2.7, whereas the 80 kDa nuclear enzyme was represented by a more diffuse band with an isoelectric pH range of 3.0–3.1. In the cytosolic extract an additional 40 kDa spot with a pI value of 3.0 resulted from partial degradation of the 80 kDa spot. In our experimental approach, only the 2-5A-free form of the enzyme was detected. It is likely that there are 2-5A-binding proteins other than those detected here, particularly the 2-5A-masked form described above and present in nuclei.

DISCUSSION

It has been generally accepted that 2-5A-dependent RNAase is either absent from or poorly represented in the nucleus. When found in nuclear fractions (Nilsen et al., 1981; St. Laurent et al., 1983), it was unclear whether it arose from cytoplasmic contamination. It is against this background that a clear-cut demonstration of 2-5A-dependent RNAase in isolated nuclei is presented here. The pertinent question of possible contamination of isolated nuclei with cytosol deserves careful attention. In this respect, proper safeguards were used in this study. A simple two-step procedure was used to purify stable nuclei. Cytoplasmic carboxylase detection together with ultrastructural observations demonstrated the effectiveness of the subcellular fractionation procedure adopted here mainly for CEM and Jurkat cell lines.

In contrast with previously reported data (St. Laurent et al., 1983), only an 80 kDa 2-5A-binding protein was found in the nuclei of CEM and Jurkat cells. In addition, the present study revealed that a large part of the nuclear 2-5A-binding site is masked and can only be determined after an unmasking procedure (Bayard and Zhou, 1992). This part of the enzyme may not be assayed by the classical radiobinding method or covalent u.v. cross-linking procedure that only measures the 2-5A-binding site in an open position, i.e. free of 2-5A. In contrast, the cytosolic fraction only contained the enzyme in a 2-5A-free form, i.e. with a 2-5A-accessible site.

In L929 cells, the level of enzyme is closely linked to the state of cell growth or confluence. An approx. 2-fold increase in 2-5A-free nuclease was observed in both the cytosolic fraction and purified nuclei during the transition from an actively growing state to a confluent growth-inhibited state. The inducibility of the nuclease as a function of cell growth is not limited to L929 cells. A similar elevated level of the enzyme was also reported (Jacobsen et al., 1983) in the JLS-V9R cell line during transition from the growing state to the confluent phase. The events responsible for its regulation remain to be elucidated. However, this induction was not an effect of cell density or membrane cell contact, as subconfluent and confluent cells had the same elevated levels. The physicochemical properties of the nuclear enzyme are found

to be slightly different from its cytoplasmic counterpart, as revealed by partial proteolysis and two-dimensional gel electrophoresis.

As observed by electron microscopy, nuclei were membraneless, and it was expected that there might be some loss of 2-5A-dependent RNAase as a result of passive diffusion during subcellular fractionation. This was not the case, as diffusion of the enzyme from the nuclei required high salt concentration. The diffusible form of the enzyme was still in a 2-5A-bound conformation, showing that the ligand co-migrates with the enzyme. There is no knowledge to date about the nature of the ligand, but, as 2-5A and 2-5A synthetase are present in the nuclei (Chebath et al., 1987; Hovanessian et al., 1987), we can expect that the 2-5A-binding site is masked by natural 2-5A. As previously suggested (Hovanessian et al., 1987), co-location of 2-5A-dependent RNAase and 2-5A synthetase might be important in regulation of the 2-5A pathway. However, it is also conceivable that the 2-5A-binding site could be associated with a nuclear protein(s) or with heterogeneous nuclear RNA in its nucleoprotein state. We have shown here that there are two distinct forms of 2-5A-dependent RNAase in different regions of the cell. Further investigations on the mechanism of translocation of the enzyme to the nucleus are required.

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