

(2'-5')Oligoadenylate in rat liver: Modulation after partial hepatectomy

(intracellular mediators/HPLC/(2'-5')oligo(A) radiobinding/growth control/interferon-induced enzymes)

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ABSTRACT (2'-5')Oligoadenylate synthetase [(2'-5')A synthetase], which synthesizes a series of oligoadenylates ppp(A2'p)_n5'A [collectively referred to as (2'-5')A], has been described previously in rat liver cells, where its concentration varied with the growth status of this organ—i.e., it decreased during the early phase of rat liver regeneration after partial hepatectomy. Because double-stranded RNA, the only known activator of this enzyme, has been detected in rat liver nuclei, (2'-5')A synthesis could occur in this tissue *in vivo*. Analysis of rat liver cell extract after HPLC by the endonuclease-based radiobinding assay revealed several components with retention times similar to (2'-5')A trimer- and tetramer-like material. A further characterization of these compounds by their susceptibility to alkaline phosphatase and snake venom phosphodiesterase, their resistance to micrococcal nuclease, and their ability to activate an endonuclease indicated the natural occurrence of oligonucleotides indistinguishable from authentic (2'-5')A in rat liver cells. Using the combination of the radiobinding assay and a simplified (2'-5')A extraction procedure that does not involve HPLC, we further show that the early drop of (2'-5')A synthetase activity during rat liver regeneration was accompanied by a similar decrease in intracellular (2'-5')A concentration. The three characteristic phases of the (2'-5')A synthetase kinetics during the first 40 hr of liver regeneration were mimicked by the kinetics of the synthesis of the (2'-5')A oligonucleotides themselves: between 6 and 20 hr after hepatectomy, there was a sharp decrease in (2'-5')A concentration; between 20 and 24 hr, the concentration of (2'-5')A reached a minimum; at 36 hr or after the first wave of DNA synthesis (the major event of liver regeneration), the (2'-5')A concentration returned to normal. In this characterization of the (2'-5')A oligonucleotide family in a functional tissue of an animal that had not been previously treated with interferon or infected with virus, the data are compatible with a physiological role of the (2'-5')A system acting as an intracellular component of the regulatory mechanisms leading to cell proliferation or differentiation.

(2'-5')Oligoadenylate synthetase [(2'-5')A synthetase] activity is present in a variety of mammalian cells and tissues (1–4) and synthesizes from ATP a series of oligomers characterized by (2'-5')phosphodiester bonds [ppp(A2'p)_n5'A with 1 < n < 18 but commonly 1 < n < 6; ref. 5] and collectively designated (2'-5')A. Their action, in cell-free systems as in intact cells, is the activation of a latent endoribonuclease (6–7) that is also widely distributed in mammalian cells and tissues (8). The ultimate result of the synthetase–nuclease pathway is the degradation of either mRNA or rRNA or both.

In vivo activity of the (2'-5')A synthetase was first documented in interferon-treated encephalomyocarditis virus (EMCV)-infected mouse L cells. In this instance, activation of

the enzyme by the double-stranded (ds) viral RNA leads to (2'-5')A synthesis (9), and the level of intracellular (2'-5')A increases in parallel with a marked degradation of EMCV mRNA (10), suggesting that the (2'-5')A system may mediate that antiviral action of interferon in this system.

Recently, a possible activation of (2'-5')A synthetase by ds regions of heterogeneous nuclear RNA of HeLa cells was reported by Nilsen *et al.* (11) and was proposed as a part of the mechanism leading to the processing of heterogeneous nuclear RNA to mRNA. The latter group also described the presence of detectable concentrations of (2'-5')A in HeLa cell nuclei and suggested that the enzyme may be active in intact nuclei (12). Moreover, independently of any previous interferon treatment, a correlation between the level of (2'-5')A synthetase activity and the growth or differentiation status of several cells and tissues has been established (2, 13–18). After partial hepatectomy, the growth-arrested cells of the remaining part of the liver regenerate. This involves a rapid and general DNA synthesis between 20 and 28 hr, followed by a first wave of mitoses (19).

The rapid decrease of (2'-5')A synthetase activity preceding the onset of DNA synthesis and its minimum level when DNA synthesis is maximal (after 24 hr) led us to propose that the level of (2'-5')A synthetase could play an important role in the mechanisms used by the cell to switch between growth or differentiation. The presence of (2'-5')A synthetase (18) and ds RNA (20) in rat liver cell nuclei further encouraged us to investigate the presence of (2'-5')A in liver and its modulation during liver cell growth after partial hepatectomy.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats [250–300 g; Animalabo (Brussels, Belgium)] were subjected to partial hepatectomy as described elsewhere (18).

Extraction of Putative (2'-5')A. Sample preparation with HPLC purification. Extracts, prepared from liver tissue (4 g) by grinding under liquid nitrogen, were heated in water (4 ml) to 100°C for 5 min and centrifuged to remove denatured proteins. Trichloroacetic acid (5% final concentration) was added, and the samples were centrifuged to remove the acid-insoluble material. The supernatant solutions were extracted six times with water-saturated ether, the extracts were lyophilized, and the resulting material was resuspended in 50 μl of 50 mM ammonium phosphate (pH 7.0) and subjected to HPLC analysis.

Sample preparation for (2'-5')A assay without HPLC puri-

Abbreviations: ds, double stranded; (2'-5')A, a series of oligoadenylates [ppp(A2'p)_n5'A] with 1 < n < 18 but commonly 1 < n < 6; (2'-5')A synthetase, (2'-5')oligoadenylate synthetase; RB, radiobinding assay; EMCV, encephalomyocarditis virus.

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fication. Liver tissue (1 g) ground under liquid nitrogen was heated in water (4 ml) to 100°C for 5 min. After centrifugation, the supernatant was treated with perchloric acid (1 M final concentration) and centrifuged to remove the acid-insoluble material. The supernatant was neutralized with KOH, and the insoluble KClO_4 pellet was eliminated by centrifugation. The (2'-5')A was precipitated from the supernatant with acetone, resuspended in 20 mM Hepes, pH 7.4/90 mM KCl and loaded on a 1-ml DEAE-cellulose column. After being washed with 30 ml of 20 mM Hepes, pH 7.4/90 mM KCl and 10 ml of water, the column was eluted with 2 ml of 450 mM $(\text{NH}_4)_2\text{CO}_3$ (21). After lyophilization, the dry residue was resuspended in 20–100 μl of H_2O . In reconstitution experiments, this procedure was found to eliminate more than 90% of the contaminating ATP [which interferes with the assay (21) at concentrations > 1 mM]. Recovery of unlabeled (2'-5')A trimers by this procedure was 75–80% in liver cell extracts.

HPLC Analysis. HPLC analysis was on a Ultrasphere ODS 5- μm column (Altex, Berkeley, CA) equilibrated in 50 mM ammonium phosphate buffer (pH 7.0) and developed with a 25-ml linear gradient consisting of 50 mM ammonium phosphate (pH 7.0) and methanol/water, 50:50 (vol/vol). Commercial AMP (Boehringer Mannheim), ATP (Boehringer Mannheim), ppp(A2'p)₂A (P-L Biochemicals), and ppp(A2'p)₃A (P-L Biochemicals) were used as standards.

Enzymatic Treatments. Micrococcal nuclease (Boehringer Mannheim) treatment was performed by incubating the sample for 15 min at 20°C in the presence of 1 mM CaCl_2 (10 $\mu\text{g}/\text{ml}$). Snake venom phosphodiesterase [Millipore Benelux (Brussels, Belgium)] treatment was performed by incubating the samples for 4 hr at 37°C with 33 units/ml. Calf intestine alkaline phosphatase (Boehringer Mannheim) action was performed by incubation of the sample for 1 hr at 37°C with 75 units/ml. The enzymatic reactions were stopped by heating the samples at 95°C for 5 min.

Determination of Extract (2'-5')A Concentration. The amount of (2'-5')A present in the rat liver was measured by the endonuclease-dependent radiobinding (RB) assay described by Knight *et al.* (21). The labeled ppp(5'A2'p)₃A3' [³²P]p5'C3'p was supplied by the Radiochemical Centre (Amersham, England). S₁₀ extract (supernatant of lysed cells obtained at 10,000 × *g*) prepared from Ehrlich ascites tumor cells grown in suspension cultures was used as a source of crude nuclease. At appropriate dilution, these preparations bind 50% of the ³²P-labeled pCp probe (5,000 cpm added per assay) after 75 min of incubation at 0–4°C in the conditions as described (21).

RESULTS

The 100 fractions (200 μl each) obtained after HPLC analysis of the acid-soluble extract from rat liver were lyophilized and then resuspended in 20 μl of 20 mM Tris·HCl (pH 7.6) and assayed for their ability to compete with ppp(5'A2'p)₃A3' [³²P]p5'C3'p for binding to the crude nuclease preparation (Fig. 1). Several substances seemed to compete in this assay. Indeed, small peaks were found at the beginning of the gradient; these correspond to the elution position of ATP, ADP, and AMP. A major, probably double, peak was eluted in the area corresponding to the (2'-5')A trimer and tetramer elution position (fractions 45–59). For further analysis, two different pools were constituted: one, corresponding to the trimer–tetramer area, consisted of an equal volume of each fraction from number 45 to 59; the second one consisted of an equal volume of each fraction from number 45 to the end of the gradient, gathering all of the oligomers present in the rat liver. Material present in pool I [putative (2'-5')A] was compared with commercial (2'-5')A

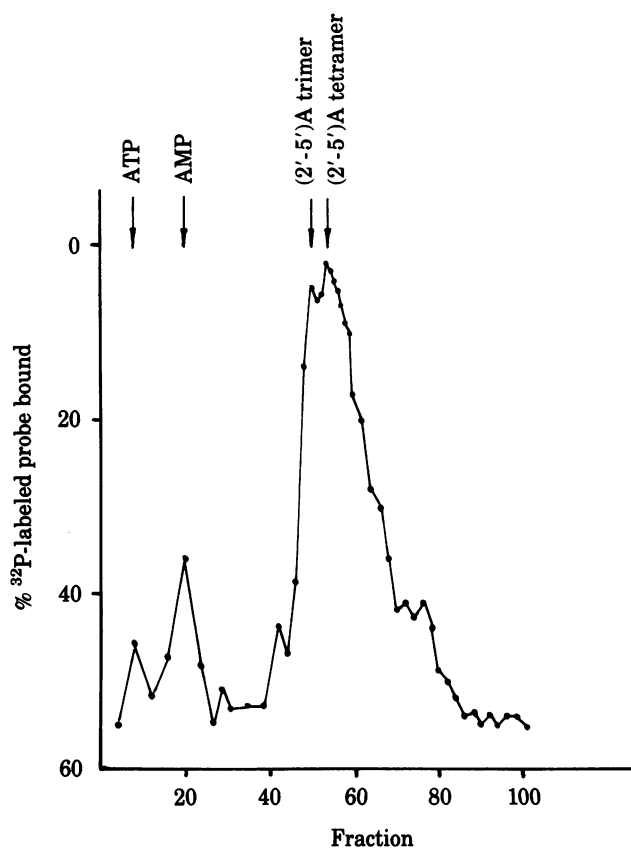


FIG. 1. Extract from rat liver tissue (50 μl) was loaded on a Ultrasphere ODS column (Altex). The column was equilibrated in 50 mM ammonium phosphate (pH 7.0) and developed with 25 ml of linear gradient consisting of 50 mM ammonium phosphate (pH 7.0) and methanol/water, 50:50 (vol/vol). Commercial AMP, ATP, trimer, and tetramer were used as markers. Fractions (100 of 200 μl each) were collected, concentrated to 20 μl , and assayed for their ability to compete with ppp(A2'p)₃A [³²P]pCp {ppp(5'A2'p)₃A3' [³²P]p5'C3'p} for its binding to the nuclease (21).

trimer by examining its response to the enzymatic action of several nucleases. The products obtained were analyzed by the radiobinding (RB) assay (Fig. 2). Based on the preliminary competition assay, pool I of putative (2'-5')A was diluted at approximately the same concentration as the commercial trimer. Characterization of pool I oligonucleotide and its enzymatically derived products was done by determining the concentration required to obtain a 50% displacement of ppp(A2'p)₃A [³²P]pCp from the crude endonuclease preparation. The following results were obtained. (i) The presence of (2'-5')A phosphodiester bonds was corroborated by the resistance of pool I to micrococcal nuclease (no modification of the activity). (ii) The drastic inhibition of activity observed after alkaline phosphatase treatment was compatible with the presence of a 5'-phosphate end in pool I. A concentration of about 300 times more core is required to displace 50% of the ³²P-labeled probe (21). (iii) Snake venom phosphodiesterase treatment of pool I resulted in a complete loss of ability to displace the radioactive probe from the nuclease. This is compatible with the cleavage of (2'-5')A phosphodiester bonds leading to the formation of ATP and AMP (21).

The biological activity of putative (2'-5')A from pool I (as well as from pool II) was evaluated by its capacity to activate the Ehrlich ascites tumor cell (2'-5')A-dependent endonuclease leading to the degradation of exogenously added [³²P]rRNA (7). In this assay, both liver (2'-5')A (pool I) and commercial trimer preparation showed the same specific biological activity—i.e.,

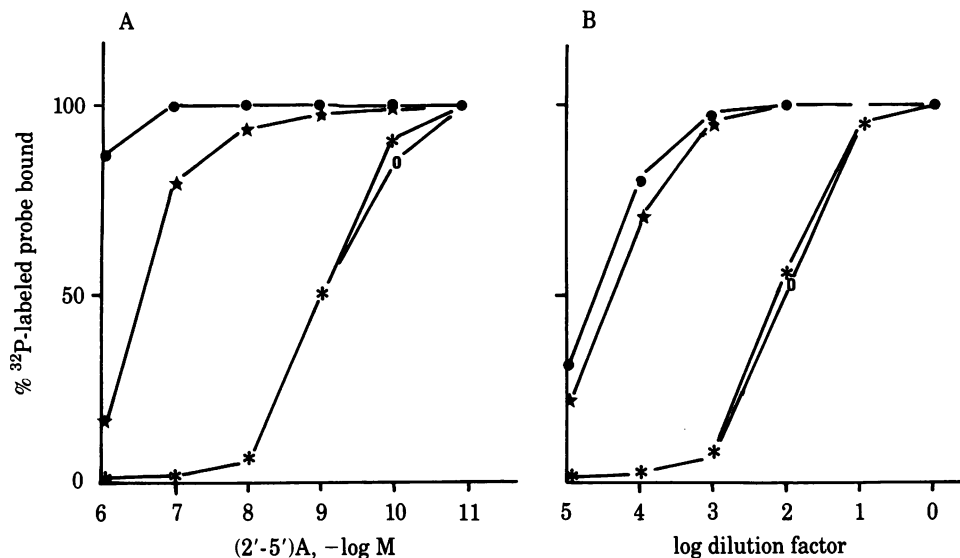


FIG. 2. Enzymatic treatments of commercial (2'-5')A (A) and putative (2'-5')A (pool I) purified from rat liver (B). 0, Without treatment; *, treated with micrococcal nuclease; ★, treated with alkaline phosphatase; and ●, treated with snake venom phosphodiesterase. The derivatives obtained after enzymatic treatment were compared for their ability to compete with ppp(A2'p)₃A [³²P]pCp {ppp(5'A2'p)₃A3' [³²P]p5'C3'p} for its binding to the nuclease (21).

50% of RNA degradation was observed for comparable concentrations of the oligonucleotides as determined by the RB assay (Fig. 3).

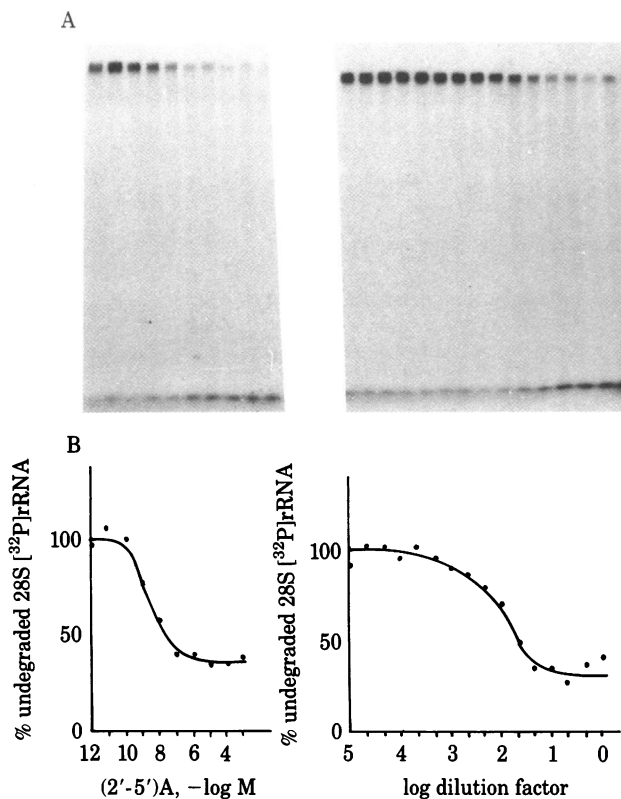


FIG. 3. (A) Activation of 28S [³²P]rRNA hydrolysis in a crude Ehrlich ascites tumor cell lysate in the presence of various concentrations of commercial (2'-5')A (Left) and the putative (2'-5')A (pool I) purified from rat liver by HPLC (Right). An autoradiogram of the dried gel is shown. Pool I was adjusted to a concentration of 1 μM as estimated by the radiobinding assay. The methodology used has been described (22). (B) Effect of commercial (2'-5')A (Left) and putative (2'-5')A (pool I) (Right) on 28S RNA hydrolysis by a crude Ehrlich ascites tumor cell lysate. The dried gel and autoradiograph were superimposed, and the area of the gel corresponding to the amount of [³²P]rRNA remaining at the origin was cut out and counted.

The total amount of (2'-5')A per gram of wet liver tissue was determined on pool II obtained after HPLC or on the fraction eluted from DEAE-cellulose arising from the treatment of 1 g of liver. The yield of this second extraction procedure was about 75–80%. A mean concentration of about 10 pmol/g of wet liver tissue was measured in 20 rats weighing between 250 and 300 g.

The first 40 hr of liver regeneration may be divided in three phases: before (i), during (ii), and after (iii) the peak of DNA synthesis. The (2'-5')A synthetase activity decreased in period i (between 6 and 20 hr after hepatectomy), remained at a min-

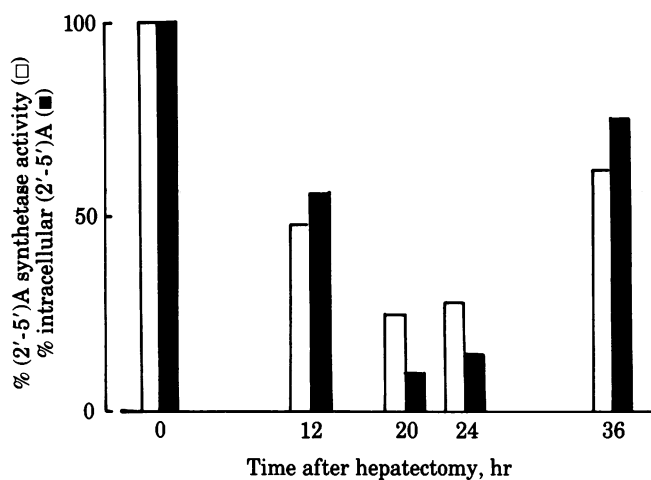


FIG. 4. Determination of (2'-5')A synthetase activity and intracellular amounts of (2'-5')A in rat liver 12, 20, 24, and 36 hr after partial hepatectomy. For each experimental animal, the two large lobes of the liver were resected as described (18), frozen in liquid nitrogen, and used as control sample. At various times after partial hepatectomy, the enzymatic activity (determined by incubation of the complex enzyme-poly(I)-poly(C) paper with tritiated ATP (17 hr at 30°C) and (2'-5')A concentration were determined in the remaining lobes. For each animal, both sets of values are expressed as a percentage of the time zero (control) value. Each point represents the average of the assays performed on six animals. A mean value of (2'-5')A synthetase activity (evaluated on 20 rats weighing between 250 and 300 g) = 500 ± 116 nmol/17 hr per g of wet liver; a mean amount of intracellular (2'-5')A = 10 pmol/g of wet liver.

imum level during elevated DNA synthesis—period *ii* (20–26 hr), and then increased—period *iii* (18). After 12 hr, the amount of (2'-5')A per g of wet tissue decreased, reaching ≈ 5 –10% of its original value between 20 and 24 hr, where it remained approximately constant, until it regained its initial level at 36 hr (Fig. 4).

DISCUSSION

The natural occurrence of (2'-5')A was established in interferon-treated and interferon-treated EMCV- or mengovirus-infected L cells (9, 21, 23), reovirus-infected (24) or EMCV-infected (25) interferon-treated HeLa cells, and in HeLa cell nuclei (12); here we clearly demonstrate its presence, mainly as trimer and tetramer, in rat liver cells. In spite of the (2'-5')A synthetase capacity to add p5'A2'p5'A to several substrates such as NAD⁺, ADP-ribose, and (A5'p)₄5'A *in vitro* (26, 27), such derivatives were not detected in extracts of interferon-treated EMCV-infected mouse L cells (21, 28). These analogues displace the radioactive probe in the RB assay at the same concentration as does (2'-5')A (28), but they do not activate the endonuclease activity except for the (A5'p)₄5'A derivatives. The loss of competing activity with the radioactive probe in RB assay after alkaline phosphatase treatment of putative (2'-5')A from rat liver indicated that these derivatives [which are resistant to alkaline phosphatase digestion (28)] did not account for the (2'-5')A displacing activity and, thus, were not present at concentrations equivalent to those of (2'-5')A. Moreover, the same concentration of pool II (containing about 10 pmol of (2'-5')A per g of wet liver) was evaluated by the RB assay and by a biological assay based on the (2'-5')A-activated nuclease degradation of ³²P-labeled RNA. These two observations excluded the presence of detectable amount of NAD⁺, ADP-ribose, and (A5'p)₄5'A derivatives of (2'-5')A in rat liver cells.

Our present observation indicates a parallel modulation of (2'-5')A synthetase activity and intracellular (2'-5')A concentration during the early phase of liver regeneration (which is characterized by an increased rate of DNA synthesis and mitosis). Two (or more) mechanisms, related to the anabolism or catabolism of the (2'-5')A oligonucleotides, may be responsible for the decrease in (2'-5')A concentration in rat liver cell extracts after partial hepatectomy. (i) The rapid drop of the (2'-5')A synthetase activity (18) could be further amplified by a decrease in liver intracellular ATP concentration. Indeed, this occurs during the first 12 hr of liver regeneration (29, 30). Nevertheless, the lowest amount of ATP (2.35 ± 0.07 mmol/g of wet liver) is equal to the highest K_m value of the rabbit or murine (2'-5')A synthetase ($2 \text{ mM} \leq K_m \leq 2.4 \text{ mM}$) (31, 32). (ii) (2'-5')A can be degraded by a specific phosphodiesterase that cleaves (2'-5')A into AMP and ATP (33–35) and by phosphatases. An increased phosphodiesterase activity was observed in growing BSC-1 cells by comparison with confluent cells (14). However, the existence of phosphatase(s) producing (2'-5')A "cores" is purely speculative at this time (36). A possible role of (2'-5')A in cell growth and differentiation has been proposed, based upon the effects observed after introduction of exogenous (2'-5')A "cores" into cells (37, 38).

The results presented here suggest a relationship between the amount of (2'-5')A and the rate of DNA synthesis after partial hepatectomy. This observation would correlate with earlier observations on mouse liver-regeneration inhibition by interferon (39), if interferon induces the (2'-5')A synthetase in rat liver as it does in mice (40). It would be of interest to investigate further what mechanisms are involved in the response to (2'-5')A in the present experimental system. It is likely that (2'-5')A acts in liver cells by activating an endonuclease because in several cell lines and tissues this protein is the only presently known

target for (2'-5')A (41, 42). What could result from a modulation of the concentration of (2'-5')A in regenerating liver? Two simple pathways may be considered. The first may involve an endonuclease-dependent heterogeneous nuclear RNA processing that leads to its maturation to mRNA and to the synthesis of protein(s) characteristic of the differentiated state of the cell. This would imply a nuclear localization of the oligonucleotide (12). The localization of the (2'-5')A synthesized in liver cells has not been defined yet, but the presence of ds RNA in this tissue (20) and the fact that more than 80% of rat liver (2'-5')A synthetase was found in the nucleus (18) seem to be compatible with such a localization. In the second eventuality, the (2'-5')A system would be a part of a larger whole cascade of inhibitors responsible for the repression of DNA synthesis of growth-arrested cells. This either could be a direct action of the activated nuclease on a step required for the G₀/G₁ phase transition to S phase or could be indirectly related to the production of other intermediates such as (2'-5')A cores.

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