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Induction of ppp(A2′p)$_n$A-dependent RNase in murine JLS-V9R cells during growth inhibition

(cell growth/endoribonuclease/interferon)

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ABSTRACT **We recently reported that interferon induces the synthesis of ppp(A2′p)$_n$A (n = 2 to ≥ 4) (2-5A)-dependent RNase in the murine cell line JLS-V9R. These cells normally contain very low levels of the nuclease; after interferon treatment, however, they develop levels approaching those found in murine L or Ehrlich ascites tumor cells. Here, we report a similar increase in the nuclease levels in JLS-V9R cells during the transition from the subconfluent actively growing state to the confluent stationary phase. Levels of 2-5A synthetase increased in parallel with the nuclease. The induced levels of both the nuclease and synthetase returned to low basal amounts after trypsinization, dilution, and culturing of the cells at subconfluent densities. The addition of anti-murine interferon (α + β) antibodies to the medium did not affect the induction of the nuclease nor could any interferon be detected in the culture supernatants as determined by the lack of antiviral activity. The increase in the enzymes was not, therefore, due to the spontaneous production of interferon. The induction of the nuclease during confluency preceded an inhibition of [^{3}H]-thymidine incorporation by the cells into DNA. The regulation of the 2-5A-dependent RNase in JLS-V9R cells may, therefore, be related to the control of cell growth.**

Interferon treatment of most animal cells induces an enzyme, ppp(A2′p)$_n$A (n = 2 to ≥4) (2-5A) synthetase, that on activation by double-stranded (ds) RNA converts ATP to 2-5A (1–3). The 2-5A in turn activates an endoribonuclease, 2-5A-dependent RNase (4–6), that degrades viral or cellular mRNA (4, 5) and also rRNA (7, 8). This pathway results in inhibition of protein synthesis in both cell-free systems (2, 3) and intact cells (7, 9, 10). When introduced into intact cells, 2-5A also inhibits DNA synthesis (10). This latter effect of 2-5A, together with the observation that monolayers of various types of cells had increased levels of 2-5A synthetase after reaching confluency (11), suggested that the 2-5A system may be involved in the interferon-mediated inhibition of cell growth; however, 2-5A could not be detected (>1 nM) in interferon-treated Daudi cells, despite their exquisite sensitivity to growth inhibition by interferon (12).

The level of 2-5A synthetase activity is considered a primary control point in the 2-5A system because in different types of cells interferon treatment increased 2-5A synthetase levels from three- to several-thousand fold (13, 14). By comparison, the 2′,5′-phosphodiesterase (15–17) that degrades 2-5A was reported to be either constant (12, 15, 18) or was moderately increased (2- to 4-fold) after interferon treatment (17, 19). Levels of 2-5A-dependent RNase were found to be unchanged (20) or increased 2-fold (12, 13, 21) after treatment with interferon; however, we have recently shown that murine JLS-V9R cells contain low basal levels of 2-5A-dependent RNase but develop nuclease levels that approach those in murine L or Ehrlich ascites tumor (EAT) cells after interferon treatment (a 10- to 20-fold increase; ref. 22). This interferon-dependent increase in 2-5A-dependent RNase was measured both by the ability of the nuclease to bind radioactively labeled 2-5A derivatives and by a functional assay in which rRNA was the substrate. Because actinomycin D inhibited induction of the nuclease by interferon in JLS-V9R, this control probably occurs at the level of transcription (22). In some systems, therefore, regulation of 2-5A-dependent RNase levels may be a second major control point in the 2-5A system, in addition to induction of synthetase activity. Here, we report an induction of the nuclease in JLS-V9R cells during the transition from a subconfluent actively growing state to a confluent growth-inhibited state. The relationship between cell growth and the 2-5A system was investigated in these cells.

MATERIALS AND METHODS

Cells and Cell Culture. The murine cell line JLS-V9R was obtained from P. A. Allen (Frederick Cancer Research Center, National Cancer Institute, Frederick, MD) (23) and then cloned (22). The JLS-V9R (clone 4) cells were used throughout and were grown at 37°C in 5% CO_2/95% air in McCoy's 5a medium supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin. Throughout the experiments, the medium was changed every 48 hr. The cells were subcultured by treatment with 0.25% trypsin and transfer to fresh medium. Cells were counted with a hemacytometer.

Preparation of Cell Extracts. The cells were harvested by scraping, washed three times in ice-cold phosphate-buffered saline, and stored as pellets at −70°C. The cells were lysed by thawing into 0.5% Nonidet P-40/90 mM potassium chloride/1.0 mM magnesium acetate/10 mM Hepes, pH 7.6. Postmitochondrial supernatant fractions were prepared by centrifugation at 12,000 × g for 10 min at 4°C. Protein concentrations were determined using the Bio-Rad protein assay (24).

Assays for the 2-5A-Dependent RNase. *The radiobinding assay (25).* Aliquots of cell extract adjusted to contain equal amounts of cell extract protein were incubated at 4°C in the presence of 5,000 cpm of ppp(A2′p)$_3$A[^{32}P]pCp (subsequently referred to as "probe") at 3,000 Ci/mmol (1 Ci = 37 GBq) unless otherwise stated. This was prepared as described previously (26) using ppp(A2′p)$_3$A (a gift from I. M. Kerr). The amount of radioactivity bound to protein was determined by its retention on nitrocellulose filters (25).

Affinity labeling of 2-5A-dependent RNase (27). A covalent

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Abbreviations: 2-5A, ppp(A2′p)$_n$A (n = 2 to ≥ 4); ds RNA, double-stranded RNA.

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linkage was formed between periodate-oxidized $p_n(A2'p)_3A$-[^{32}P]pC (n = 1–3) and the nuclease in cell extracts by incubation under reducing conditions by using the procedure of Wreschner *et al.* (27) with the modification described previously (22). Proteins were separated by NaDodSO$_4$/10% polyacrylamide gel electrophoresis and the ^{32}P-labeled proteins were visualized by autoradiography on Kodak SB-5 x-ray film.

The rRNA cleavage assay (8). The cell extracts were incubated for 2 hr at 30°C in the presence or absence of 1.0 μM tailed 2-5A tetramer triphosphate, an active phosphodiesterase-resistant analog of 2-5A (ref. 28, a gift of P. F. Torrence) under modified protein synthesis conditions (8). The RNA was isolated (13) and applied to a glyoxal/1.8% agarose gel (8, 29).

Assay of 2-5A Synthetase. The synthetase present in the postmitochondrial supernatant fractions was bound to poly(I)·poly(C)-cellulose and incubated with 4.0 mM ATP by the method of Stark *et al.* (30). The 2-5A synthesized by the bound enzyme was determined using the radiobinding method of Knight *et al.* (25).

Incorporation of [^{3}H]Thymidine into DNA in JLS-V9R Cells. DNA was labeled by incubation in medium containing [^{3}H]thymidine (Amersham, 51 Ci/mmol) at 1 μCi/ml for 1 hr at 37°C. The cells were harvested and washed twice with 2.0 ml of phosphate-buffered saline containing about 20 μM unlabeled thymidine. The cells were then suspended in 2.0 ml of phosphate-buffered saline, 1.0 ml of the cell suspension was centrifuged at 10,000 × g for 10 min, and the resulting cell pellet was used to determine nuclease levels by the radiobinding assay. The cell number was determined by counting a 1:100 dilution of the cell suspension using a hemacytometer. From the remaining cell suspension, 200 μl was added to 1.0 ml of 5% trichloroacetic acid at 0°C and the incorporation of [^{3}H]thymidine into DNA was determined from this material so described (12).

RESULTS

2-5A-Dependent RNase and 2-5A Synthetase Levels Increase in Confluent Monolayers of JLS-V9R Cells. Stark *et al.* (11) have reported that the level of 2-5A synthetase increased in various cell lines grown in monolayer culture during confluency. That finding showed that conditions other than interferon treatment could regulate the cellular level of the enzyme. Recently, we reported that both the 2-5A-dependent RNase and 2-5A synthetase are inducible by interferon in murine JLS-V9R cells (22). It was of interest, therefore, to determine whether the level of the nuclease fluctuates as a function of cell growth. 2-5A-dependent RNase levels were measured by a specific radiobinding assay that uses ppp(A2'p)$_3$A[^{32}P]pCp as a probe (25, 26). 2-5A-binding activity (presumptive nuclease) increased in parallel with 2-5A synthetase activity as the cells proceeded from a subconfluent actively growing state to a confluent stationary state (Fig. 1). In subconfluent cells (measured <50 hr after subculturing of a confluent cell layer), 4% of the probe was bound. In contrast, 27% binding was observed in late-confluent cells (Fig. 1*B*), while extracts from interferon-treated (2,000 units/ml) JLS-V9R cells or from untreated murine L cells bound 35% and 55% of the probe (22), respectively. The binding was specific for 2-5A because it was abolished by the addition of unlabeled 2-5A (250 nM) to the assay mixtures (data not shown). The cells used in the experiment shown in Fig. 1 reached confluency at about 50 hr. The growth rate was greatly reduced after 50 hr, and the cells ceased to divide after 100 hr in culture (Fig. 1*B*). The largest increase in nuclease levels occurred between 50 and 100 hr. In addition, 2-5A synthetase increased during confluency (Fig. 1*A*), with peak levels that were 46-fold those at the onset of the experiment.

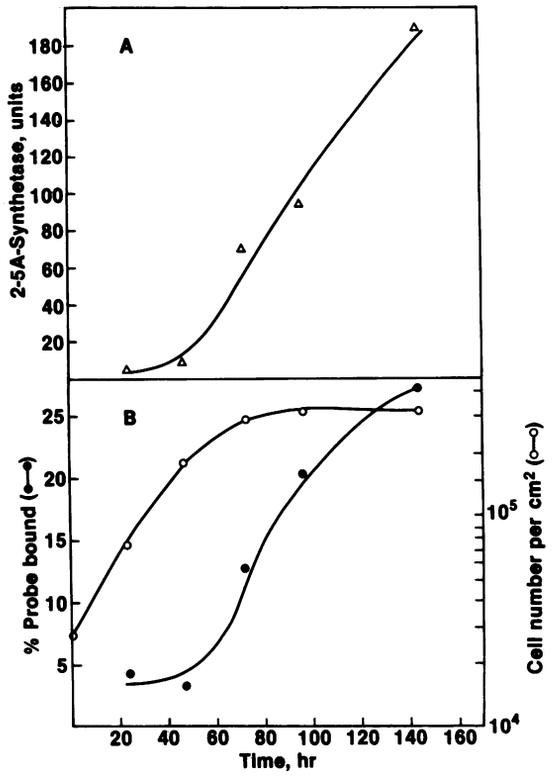

FIG. 1. Induction of 2-5A synthetase (*A*) and 2-5A-dependent RNase (B) in murine JLS-V9R cells during cell growth inhibition (*B*). At various times, the cells were harvested, cell numbers were determined (○), and cell extracts were prepared. 2-5A synthetase (△) was assayed by using poly(I)·poly(C) bound to cellulose and 2-5A-dependent RNase (●) was assayed by the radiobinding assay. The cells become confluent at about 50 hr.

Addition of the protease inhibitor phenylmethylsulfonyl fluoride to the cell lysis buffer had no effect on the levels of nuclease measured in either subconfluent or confluent cells (unpublished data). Therefore, inactivation of the nuclease by a phenylmethylsulfonyl fluoride-sensitive protease in rapidly growing cells, analogous to that recently reported in NIH 3T3 (Moloney murine leukemia virus) cells (31), cannot account for the observed differences in probe binding activity.

The interferon and ds RNA-stimulated protein kinase (32–34) was not detectable in extracts of untreated subconfluent or late-confluent JLS-V9R cells (unpublished data). The kinase was, however, detected in JLS-V9R cells after interferon treatment (22).

Affinity Labeling of the 2-5A-Dependent RNase. Although radiobinding activity is relatively specific for the 2-5A-dependent RNase, an affinity labeling technique was used to determine whether substantial levels of other 2-5A-binding proteins also existed in JLS-V9R cells. The method consists of covalently linking a ^{32}P-labeled derivative of 2-5A to the 2-5A-binding protein(s) followed by analysis of the ^{32}P-labeled proteins on NaDodSO$_4$/10% polyacrylamide gels (27). A 2-5A-binding protein of 77,000–85,000 daltons (subsequently referred to as p80) was reported in two studies (27, 35) to copurify with 2-5A-dependent RNase; we assume here that p80 and the nuclease represent the same protein. An extract of EAT cells was included in the study for comparison (Fig. 2, lanes 1 and 2). A gradual increase in p80 was observed in JLS-V9R cells between 56 and 150 hr in culture—i.e. after confluency (Fig. 2). This result is consistent with the radiobinding data in Fig. 1*B*. Furthermore, the only material labeled by the 2-5A probe was p80, the pre-

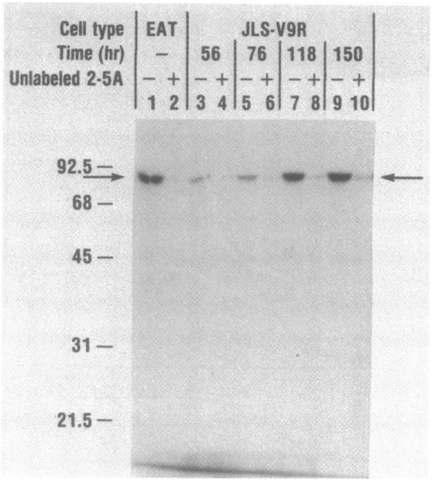

FIG. 2. Affinity labeling of the 2-5A-dependent RNase with p_n-$(A2'p)_3A[^{32}P]pC$ (n = 1–3). Nuclease present in the postmitochondrial supernatant fractions from EAT (lanes 1 and 2) and JLS-V9R (lanes 3–10) cells was covalently bound to the radioactive 2-5A derivative. Assays were carried out in the presence (+) or absence (−) of unlabeled trimer 2-5A (250 nM) as indicated. Proteins were separated on NaDodSO$_4$/10% (wt/vol) polyacrylamide gels. The JLS-V9R cells were grown in monolayer culture for the times indicated. The positions of the protein markers (numbers to the left represent their molecular weight × 10^{-3}) and the presumptive 2-5A-dependent RNase (arrow) are indicated. An autoradiograph of the dried gel is shown.

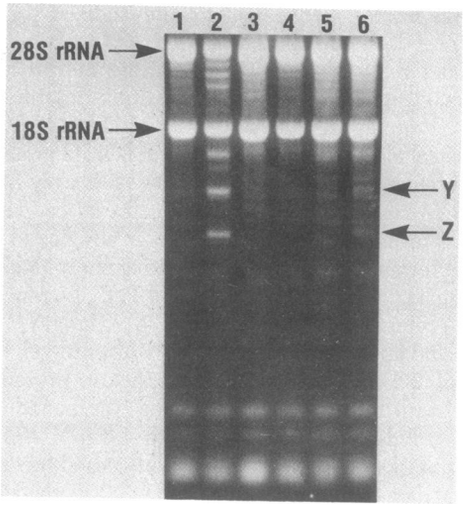

FIG. 3. 2-5A-mediated cleavage of rRNA in cell-free systems. Postmitochondrial supernatants were incubated for 2 hr at 30°C with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) 1.0 μM tailed 2-5A tetramer triphosphate. The RNA was then isolated (13) and applied to a glyoxal/1.8% agarose gel (29). Cell extracts were from untreated murine L cells (lanes 1 and 2), subconfluent JLS-V9R cells (lanes 3 and 4), and late-confluent JLS-V9R cells (lanes 5 and 6). Arrows indicate positions of the 18S and 28S rRNAs and the 2-5A-mediated 18S rRNA products (Y and Z) (36). A photograph of an ethidium bromide-stained gel is shown.

sumptive nuclease. Labeling of p80 was specifically prevented by addition of unlabeled 2-5A (250 nM) to the assay mixtures (Fig. 2, lanes 2, 4, 6, 8, and 10).

Induction of Functional 2-5A-Dependent RNase During Confluency. Activation of the 2-5A-dependent RNase in intact cells and in cell-free systems results in a specific cleavage pattern of rRNA (8). Previously, we used the rRNA cleavage assay to show that protein p80 and 2-5A-dependent RNase activity increase in parallel in interferon-treated JLS-V9R cells (22). We sought to determine whether 2-5A-mediated rRNA cleavage could be detected in cell-free systems from JLS-V9R cells at different stages of cell growth. For reference, the 2-5A-mediated cleavage products Y and Z from a cell-free system from untreated murine L cells are shown in Fig. 3 (lane 2). There was no detectable 2-5A-dependent RNase activity in response to added 2-5A in extracts from the subconfluent JLS-V9R cells (Fig. 3, lane 4). In contrast, addition of 2-5A to a cell-free system from late-confluent JLS-V9R cells resulted in the appearance of the Y and Z fragments, although the amount of rRNA cleavage was much less than that observed in the cell-free system from L cells (compare lanes 6 and 2). Radiobinding assays (described above) indicated that the nuclease level in late-confluent JLS-V9R cells was about half of that found in control murine L cells. The nuclease levels in the late-confluent JLS-V9R cells, however, appear to be substantially less as estimated by the rRNA cleavage assay (Fig. 3) as compared with the radiobinding and affinity labeling assays (Figs. 1 and 2, respectively). The reason for this discrepancy is not known but it may result from the presence in growth-arrested JLS-V9R cells of material inhibitory to nuclease activity.

JLS-V9R Cells Do Not Spontaneously Produce Interferon. Cell lines that spontaneously produce interferon have been reported (37). Because the nuclease is interferon-inducible in JLS-V9R cells (22), the possibility that the induction of the nuclease in confluent JLS-V9R cells resulted from a spontaneous production of interferon was investigated. However, no antiviral activity (using murine L cells and encephalomyocarditis virus) was found in the culture supernatants from late-confluent JLS-V9R cells (unpublished data). Moreover, growth of JLS-V9R cells in medium containing sheep anti-murine interferon (α + β) globulin did not prevent the increase in the nuclease in the confluent cells (Fig. 4).

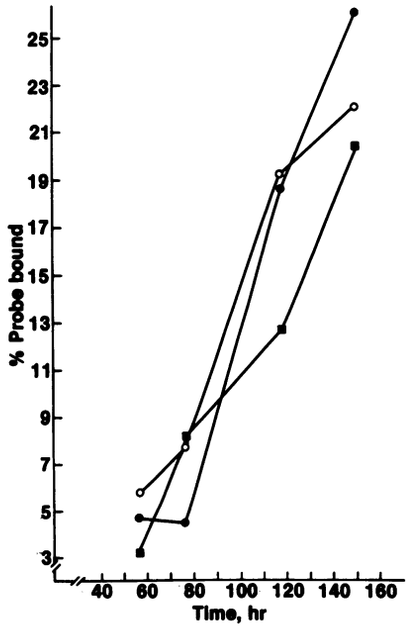

FIG. 4. Anti-murine interferon (α + β) globulin does not prevent the induction of 2-5A-dependent RNase during confluency. JLS-V9R cells were grown from the subconfluent to late-confluent state with no additions (●) or with 1:1,000 dilutions of either normal sheep globulin (○) or sheep anti-murine interferon (α + β) globulin (■). The cells were harvested at the times indicated and 2-5A-dependent RNase levels were measured by the radiobinding assay. Confluency occurred at about 70 hr. The sheep anti-murine interferon (α + β) globulin (a gift from I. Gresser) had a neutralizing titer of 1:400,000 against 8 units of C-243 cell mouse interferon.

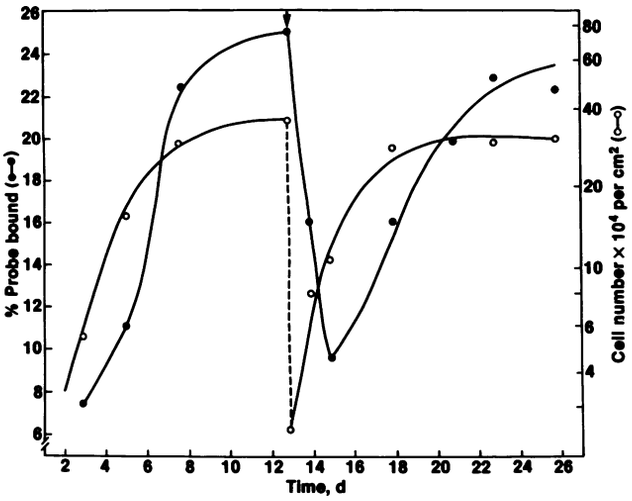

FIG. 5. Levels of 2-5A-dependent RNase during two consecutive growth cycles. JLS-V9R cells were grown from subconfluency to late-confluency (day 13, end of first cycle) and then trypsinized, diluted 1:20 in fresh medium, and subcultured (arrow). The cells became confluent on day 5 and again on day 17. The end of the second growth cycle was taken to be day 26. At the indicated times, the cells were harvested. The cell numbers (○) were determined and the level of 2-5A-dependent RNase (●) was measured by the radiobinding assay. Cellular 2-5A synthetase levels were 12.5, 223.0, 18.0, and 35.7 units (pmol of 2-5A per hr per mg of protein) on days 3, 13, 14, and 18, respectively.

Nuclease Levels in Late-Confluent JLS-V9R Cells Decrease When the Cells Are Subcultured at a Lower Cell Density. Cultures of JLS-V9R cells were grown to late stationary phase (late-confluency), trypsinized, and subcultured at a 1:20 dilution in fresh medium. 2-5A-binding activity (presumptive nuclease) was then monitored throughout two consecutive growth cycles (Fig. 5). In the first cycle of growth, the level of binding activity increased dramatically after the cells became confluent on day 5 and reached maximal levels during late stationary phase (day 12). 2-5A-binding activity decreased over a 2- to 3-day period after dilution to a lower cell density (Fig. 5, the arrow indicates the time of dilution). This level of 2-5A-binding activity was about 35% of that observed prior to subculturing. During the second growth cycle, an increase in probe binding was observed when the cells again became confluent on about day 17. A similar cyclic fluctuation in the level of the 2-5A synthetase was also observed (Fig. 5). A more detailed analysis of the kinetics of the decrease in 2-5A-binding activity after subculturing is shown in Fig. 6. The largest decrease occurred between 8 and 36 hr, even though cell division did not occur during the first 24 hr after subculturing. Two cycles of cell division were observed between 24 and 48 hr.

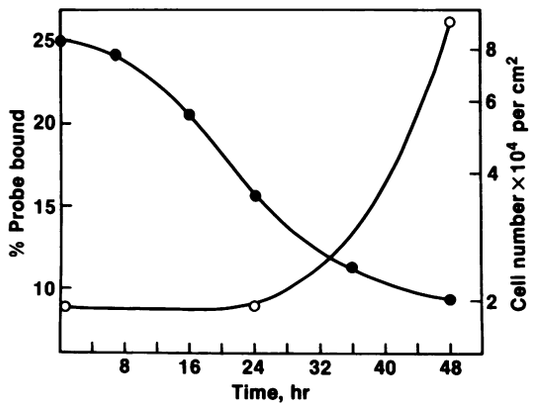

FIG. 6. Kinetics of declining 2-5A-dependent RNase levels after subculturing. JLS-V9R cells were grown to late confluency, trypsinized, diluted 1:20 in fresh medium, and subcultured for the times indicated. The cell numbers (○) were determined and the levels of 2-5A-dependent RNase (●) were measured by the radiobinding assay.

Table 1. Induction of the nuclease precedes the inhibition of [^{3}H]thymidine incorporation into DNA in JLS-V9R cells

Time, hr	Cells, no. × 10^{-4} cm^2	[^{3}H]Thymidine incorporation*	% probe bound
0	5.8	30,190	1.6
24	9.0	35,566	4.4
48†	19.6	41,990	11.1
72	22.8	37,019	14.6
96	25.8	16,692	14.0
120	27.7	13,491	14.2
144	28.4	3,877	16.8

The radiobinding assay was carried out using 300 μg of cell extract protein. The probe used was at a lower specific activity than was used in Figs. 1, 4, 5, and 6. This resulted in a 30–50% lower binding of probe to extracts of L, EAT, and JLS-V9R cells.
* cpm per 10^6 cells.
† Time at which the cells became confluent and growth was inhibited.

The decrease in 2-5A-dependent RNase after subculturing is not due to the process of subculturing itself, including trypsinization, addition of fresh medium, or new culture flask surface. Cell cultures were also trypsinized and diluted 1:2, 1:5, 1:10, and 1:20 in fresh medium; 2-5A-binding activity was measured 24 hr later. Although all of the subcultures were similarly treated, only those cultures diluted at least 1:10 showed a marked decrease in the 2-5A-binding activity (data not shown).

Induction of the Nuclease Precedes Inhibition of [^{3}H]Thymidine Incorporation into DNA. The induction of the nuclease levels during confluency may be either a cause or a consequence of the inhibition of cell growth. To approach this problem, we determined the kinetics of the rise in the nuclease levels in cells labeled with [^{3}H]thymidine (Table 1). The cells became confluent after about 48 hr while the nuclease as measured by the radiobinding assay reached nearly maximal levels by 72 hr. A marked decrease in the incorporation of [^{3}H]thymidine into DNA occurred between 96 and 144 hr, which therefore followed the induction of the nuclease by at least 24 hr. The results are consistent with a role for the 2-5A-dependent RNase in the inhibition of cell growth in JLS-V9R cells.

DISCUSSION

Here we report that the level of 2-5A-dependent RNase is closely linked to the rate of cell growth during confluency in JLS-V9R cells. The nature of the agents or events responsible for the regulation of the nuclease levels remains to be elucidated. Induction of the enzyme by interferon probably can be excluded: interferon, as measured by antiviral activity, was not found in culture supernatants from late-confluent cell monolayers and addition of anti-interferon ($\alpha + \beta$) serum to the medium did not prevent the induction (Fig. 4). The interferon assay used detected <5 units per ml whereas >500 units per ml of interferon were required to induce the nuclease in JLS-V9R cells to the levels observed in late-confluent cells (22). Also, addition to subconfluent JLS-V9R cells of murine interferon-γ (a gift of S. Vogel, Uniformed Services University of the Health Sciences) at 100 units/ml or less failed to induce the nuclease (data not shown).

Interferon treatment of either the subconfluent or late-confluent JLS-V9R cells resulted in similar levels of 2-5A-dependent RNase, so that the relative induction by interferon was

less in late-confluent cells (unpublished data). The level of the nuclease in untreated late-confluent cells was about 75% of that seen after treatment of these cells with interferon at 2,000 units/ml (this paper and ref. 22). In contrast, the level of 2-5A synthetase found in the late-confluent cells was about 1% of that seen after interferon-treatment (22). Therefore, the level of the nuclease reached nearly maximal values during late confluency whereas 2-5A synthetase was at only about 1% of its potential maximal level under the same conditions.

The induction of the nuclease was probably not entirely an effect of cell density. Subconfluent JLS-V9R cells cultured in serum-free medium showed elevated (2-fold) levels of the nuclease as compared with subconfluent control cells cultured in the presence of serum (data not shown). Furthermore, the inducibility of the nuclease as a function of cell growth is not limited to JLS-V9R cells. An approximate 2-fold increase in the nuclease was observed during confluency in murine L cells, in PSA-5E cells (a differentiated murine teratocarcinoma cell line), and in Vero cells (green monkey kidney cells) (data not shown). Clearly, however, the JLS-V9R cells are unusual in the extent to which the nuclease levels are regulated. These results, together with our previous findings on the inducibility of 2-5A-dependent RNase by interferon (22), suggest that regulation of the nuclease in JLS-V9R cells is complex and that its importance may extend to mechanisms related to the control of cell growth.

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