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REGULATION OF 2-5 A SYNTHETASE ACTIVITY AND ANTIVIRAL STATE IN INTERFERON TREATED CHICK CELLS.

M. Lab[1], M.N. Thang[2], K. Soteriadou[3], F. Koehren[1], J. Justesen[2].

1) Faculté de Médecine, Laboratoire de Virologie
et Groupe de Recherches de l'INSERM U 74
3, rue Koeberlé 67000 STRASBOURG France.
2) Institut de Biologie Physico-Chimique
13, rue Pierre et Marie Curie 75005 PARIS France
3) Institut Pasteur Hellenique
127 Vassilissis Sophia Avenue ATHENS Greece.

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SUMMARY - The interferon induced 2'5' oligoadenylate synthetase activity in chick embryo fibroblasts is transient and decays in the absence of interferon. Inhibition of cell protein synthesis by cycloheximide after removal of IFN prevents this decay ; after 2 5 A synthetase activity has decayed, addition of cycloheximide causes recovery and "superinduction" of the 2-5 A synthetase activity. The variations in 2-5 A synthetase activity are correlated with similar variations in antiviral state. These results may be accounted for by translational and post translational regulation of 2-5 A synthetase activity in chick embryo fibroblasts.

INTRODUCTION

The intracellular antiviral response induced by interferon is mediated by two major pathways : phosphorylation of the initiation factor eIF-2 by a protein kinase and synthesis of 2'5' oligoadenylates by 2'5' oligoadenylate synthetase (2-5 A synthetase), both enzymes being dependent on double stranded RNA (1-4). In primary chick embryo fibroblasts (CEF), interferon (IFN) induces high levels of 2-5 A synthetase activity (5-7) whereas no specifically induced protein kinase activity has been found (7). Thus, the 2-5 A synthetase pathway seems until now to constitute the only known mechanism by which the intracellular response to IFN is mediated in CEF. We previously reported (8-9) that the antiviral state (AVS) in CEF is transient and decays in the absence of IFN. Inhibition of cell protein synthesis by cycloheximide immediately after removal of IFN prevents this decay. After AVS has decayed, addition of cycloheximide causes full recovery within 24 hours of the apparently lost AVS. Here we describe the fate of

Abbreviations : CEF : chick embryo fibroblasts ; Interferon : IFN ; 2'5' oligoadenylate synthetase : 2-5 A synthetase ; Antiviral state : AVS ; polyinosinic acid : poly (I) ; polycytidilic acid : poly (C).

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2-5 A synthetase activity in CEF after removal of IFN under similar experimental conditions. Our results show that 2-5 A synthetase activity in IFN-treated CEF is transient and dependent on the presence of IFN in the culture medium. Inhibition of protein synthesis for appropriate intervals, after the removal of IFN, can result in the maintenance, the recovery or even the"superinduction"of 2-5 A synthetase. Such results may be accounted for by translational and post translational regulation of 2-5 A synthetase activity in CEF.

MATERIALS AND METHODS

Cells

Monolayers of primary CEF were prepared by trypsination of 10 or 11 day old embryos seeded in 30 ml plastic flasks (5.10^6 cells). Eagle's medium MEM (Eurobio) supplemented with 10 or 2% heated calf serum was used as growth or maintenance medium respectively.

Interferon treatment

Monolayers of primary CEF (5×10^6 cells) in plastic flasks (30 ml) were treated at 37°C with 300 U ml^{-1} of crude chick IFN in Eagle's medium (MEM Eurobio) 2% calf serum (Flobio) as previously described (9).

2-5 A synthetase activity assay

Cells were washed (3 times, 3 ml) in buffer A containing 35 mM Tris HCl pH 7.5, 140 mM NaCl, 3 mM Mg $(OAc)_2$. The cells were lysed in 0.4 ml of buffer B containing 20 mM Tris pH 7.5, 5 mM Mg $(OAc)_2$, 120 mM KCl, 1 mM dithiothreitol, 10% glycerol and 0.5% NP 40. After 10 minutes at 4°C the cell homogenates were scraped off and centrifuged at 10000 g for 10 min. 15 µl of the supernatant was applied to 15 µl poly (I) poly (C) agarose (Choay, France) with 50 µl buffer D containing 20 mM Tris HCl pH 8.0, 5 mM Mg $(OAc)_2$, 25 mM KCl, 0.5 mM dithiothreitol, 1 mM EDTA and 10% glycerol (v/v) and left at room temperature for 15 min. The agarose beads were washed twice with 500 µl buffer D. To the pellet of agarose was added 10 µl of a mixture to give a final concentration of 5 mM ATP (Ca 0.02 µCi $\alpha(^{32}P)$ ATP), 20 mM Mg $(OAc)_2$, 12 µg/ml Poly (I) poly (C), 0,25 mg/ml bovine serum albumine in buffer D (10-11). After two hours of incubation at 37°C the reaction was stopped by addition of 20 µl mM EDTA 6 µl of reaction mixture was applicated on polyethylene-imine cellulose thinlayer plates chromatographed in 2 M Tris-HCl pH 8.6. Radioactive spots were localized on the chromatogram using X ray film, cut out, and radioactivity was determined by Cerenko countings. The percentage conversion of ATP into 2'5' oligoadenylates was used to calculate the activity in units of nanomoles ATP incorporated per hour per mg protein. Protein concentration is determined according to Spector (11).

Determination of the antiviral state

Cells were washed 3 times with PBS and infected at a multiplicity of 1 with Sindbis virus. After 1 hour of adsorption, cells were washed and incubated in 5 ml fresh medium (MEM 2% calf serum) containing actinomycin D (Sigma) 0.5 µg ml^{-1} for 18 hours at 37°C. Cultures were then frozen at -80°C thawed and the viral yields was determined by plaque assay. Control monolayers without IFN were included for each assay, infected and titrated similarly. Cell antiviral state was expressed by the log 10 inhibition of virus yield in IFN-treated cells compared with untreated cultures.

RESULTS AND DISCUSSION

2-5 A synthetase activity in CEF appeared during the first hour of IFN treatment and reached its maximum level in 5-6 hours (fig. 1a). The

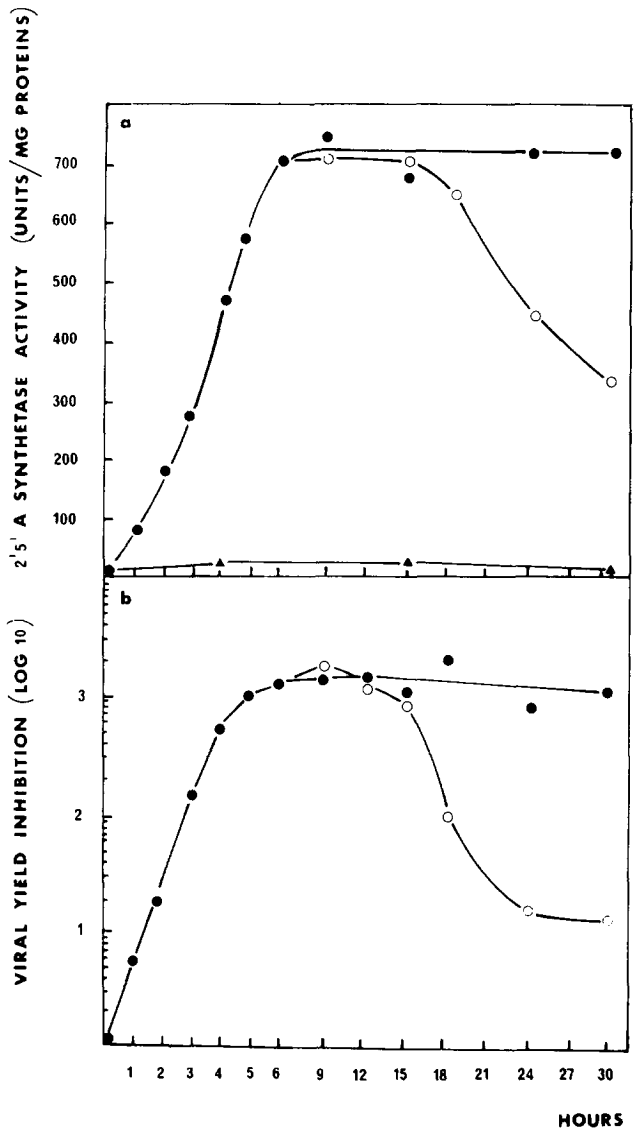

FIGURE 1 : a) 2-5A synthetase activity and b) antiviral state in CEF. Time-course of appearance and decay.
Monolayers of primary CEF (5 x 10^6 cells) in plastic flasks (30 ml) were treated at 37°C with 300 U ml^{-1} of crude chick IFN in Eagle's medium 2% calf serum for different lengths of time until 30 hours (●), or for 6 hours and then washed 3 times with PBS and replenished with MEM 2% calf serum (○). 2-5 A synthetase activity in control cells extracts (▲).

AVS as measured by virus yield inhibition of Sindbis virus appeared with similar kinetics (fig. 1b). The 2-5 A synthetase and the AVS were maintained as long as interferon was present in the culture medium. After the removal of IFN the AVS and the 2-5 A synthetase activity were maintained for 9-10 hours and thereafter they fell within about 14 hours. The decrease of AVS was more rapid than the decay of 2-5 A synthetase. This may be due to the

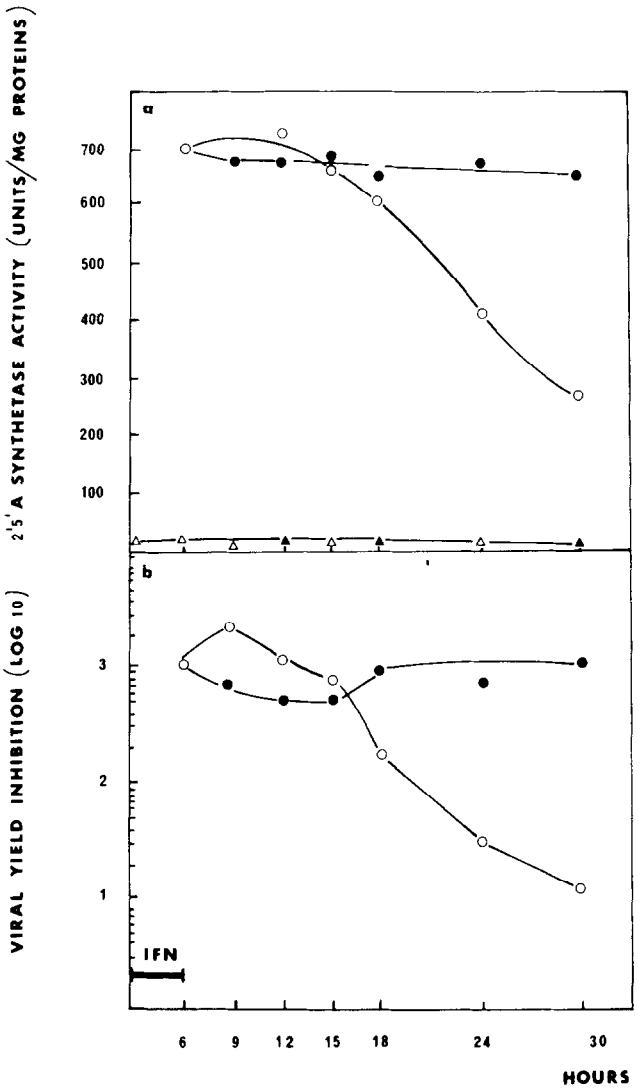

FIGURE 2 : Maintenance of 2-5 A synthetase activity and AVS by cycloheximide addition after IFN removal.
a) 2-5 A synthetase activity and b) viral yield inhibition were measured under the conditions described in Material and Methods. After a 6 hour treatment with IFN (300 U ml^{-1}) CEF cultures (5.10^6 cells) were washed three times with PBS and replenished with 5 ml Eagle's medium (MEM) 2% calf serum with (●) or without (O) cycloheximide (Sigma, 20 µg ml^{-1}). At the indicated times enzyme assays were carried out for 2-5 A synthetase activity and cell monolayers were infected after three washes with PBS in order to eliminate inhibitor. 2-5 A synthetase activity in control cell with (▲) or without cycloheximide (Δ).

fact that comparisons are being made between enzymatic activities in cell extracts and viral yield inhibition observed after the infection of cultures of growing cells. During the first hours of infection, enzyme formation or degradation can still occur. Thus, the viral yield inhibition seems to anticipate the general trend of 2-5 A synthetase activity.

When cycloheximide (20 μg ml^{-1}) was added immediately after removal of IFN, we observed (fig. 2) that virus yield inhibition as well as 2-5 A synthetase activity were maintained at the same level for 24 hours. The decrease of 2-5 A synthetase activity in the absence of a protein synthesis inhibitor suggests that it is under the control of a regulatory protein. The means of regulation may be either degragation of the enzyme or reversible inactivation.

Consequently we determined whether the 2-5 A synthetase could be recovered after a decay period. After 6 hours of IFN treatment the cells were left without IFN for 24 hours, and cycloheximide added at 30 hours (Fig. 3). We noticed as before that removal of IFN led to the decay of 2-5 A synthetase activity and the AVS. Following addition of cycloheximide at 30 hours, the 2-5 A synthetase in cell extracts was found to increase after a lag period of 12 hours and to reach after 24 hours of cycloheximide treatment the same level as found initially at the 6th hour when IFN was removed. These results suggest that an unstable protein reversibly inactivates the 2-5 A synthetase activity.

Furthermore, in the same experiments (Fig. 3) removal of cycloheximide after 18 hours gave rise to an additional increase in 2-5A synthetase activity and a transient increase in virus yield inhibition. This may be explained by the accumulation of mRNA for 2-5 A synthetase during the treatment with cycloheximide. Inhibition of mRNA synthesis by actinomycin after the removal of cycloheximide did not affect the level of 2-5 A synthetase activity but resulted in the maintenance of the AVS at its peak. Again the decline in AVS in the absence of actinomycin D could anticipate a subsequent fall in synthetase activity but experiments have not yet been carried out to investigate this.

It has already been observed that appropriate treatments with metabolic inhibitors stimulate the induction of specific enzyme activity (13) or IFN production (14, 15). Stimulation of the AVS by actinomycin D has been reported (16). It has also been shown that chick cell maintained AVS for a longer period of time when enucleated (17). Similarly we have recently found that during the maturation of the red cell in rabbits, 2-5 A synthetase remains stable for several days in the reticulocyte after enucleation (Ferbus, Justesen, Bertrand, Thang, unpublished results). These observations suggest the existence of a mechanism of regulation of AVS requiring mRNA synthesis and mediated by an unstable protein (s). The present study shows that AVS and 2-5 A synthetase activity in CEF are coordinately regulated. Once induced by IFN, the mRNA coding for 2-5 A synthetase still appears functional 42 hours after the end of IFN treatment as indicated by the additional increase of 2-5 A synthetase activity after removal of cycloheximide (fig. 3). This mRNA

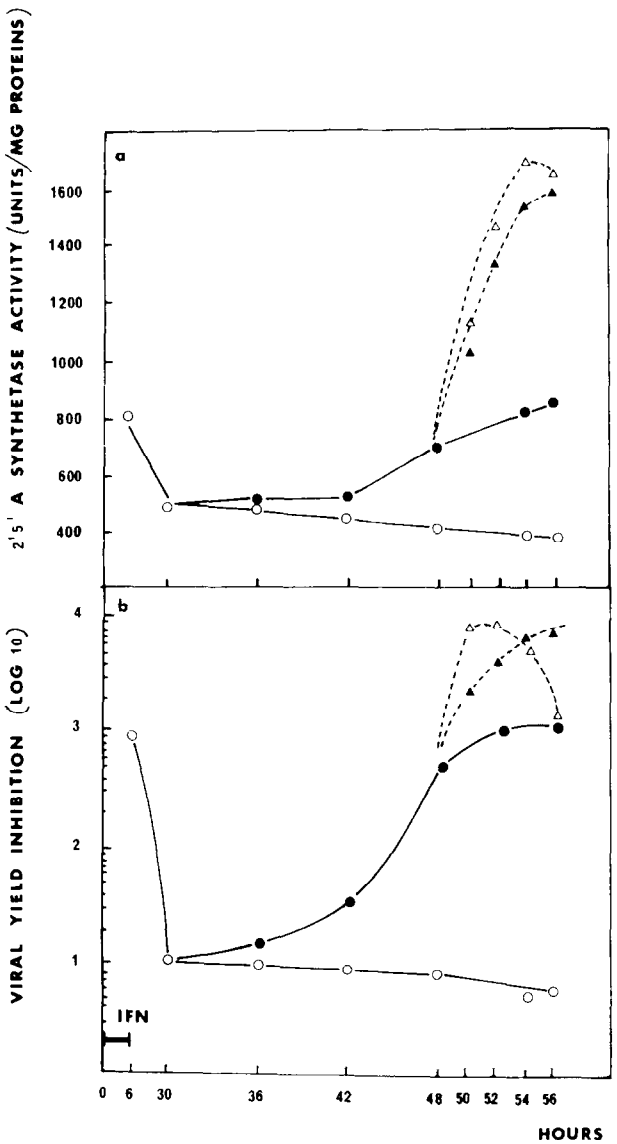

Figure 3 : Recovery and "superinduction" of 2-5 A synthetase and AVS by cycloheximide.
a) 2-5 A synthetase activity
b) antiviral state.
Monolayers of CEF (5.10 cells) were treated 6 hours with 300 U ml^{-1} of IFN. Cultures were then washed 3 times with PBS and 5 ml of Eagle's medium (MEM) 2% calf serum were added for 24 hours. Culture medium were then changed with cycloheximide (20 µg/ml) (●) or without (○) ; after 18 hours of cycloheximide treatment, some cultures were washed 3 times with PBS and replenished with medium with (▲) or without (△) the addition of Act D 0,5 µg ml^{-1}.

may be transiently inactivated by factors that need cellular protein synthesis The recovery of its activity under cycloheximide treatment indicates that its activity could be regulated in a reversible manner.

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