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THE 2'5' OLIGOADENYLATE SYNTHETASE HAS A MULTIFUNCTIONAL 2'5' NUCLEOTIDYL-TRANSFERASE ACTIVITY

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SUMMARY - The interferon induced 2'5' oligoadenylate synthetase catalyzes the synthesis of a series of 2'5' heteronucleotides with the general structure of $pppA(pA)_npN$ and NAD-NMP. The enzyme also catalyzes the 2' adenylation of tRNA. The requirement for the acceptor site is an AMP group linked in a RpA configuration where R stands for pyrophosphate, NAD^+, oligomeric or polymeric primers. NAD^+ and tRNA are found to be inhibitors for the synthesis of 2'5' oligoadenylates. Hence, it is suggested that NAD^+ and tRNA could play a role in the regulation of the synthesis of 2'5' oligoadenylates in the cell. The name of 2'5' nucleotidyl-transferase is proposed for this multi-functional enzyme which is not merely an ATP polymerase.

INTRODUCTION

The 2'5' oligoadenylate synthetase (1) is a cellular enzyme that catalyzes the synthesis of a series of oligoadenylates caracterized by a 2'5' phosphodiester linkage with the general structure $pppA2'(p5'A)_n$, $n \geqslant 1$. The level of the activity of this enzyme in cells varies with cell types, cell tissues, conditions of growth etc (2-4). It can be markedly increased by treatment of cells with interferons (5-8). It is generally thought that the enzyme is involved in the antiviral action of interferons through the control of a nuclease system (9-18) which is particularly activated by the 2'5' oligoadenylates. However, there is no direct evidence proving this mechanism responsible for the inhibition of virus replication in interferon treated cells (19-20), even though the presence of 2'5' oligoadenylates has been detected in cells infected by virus and treated with interferons (21). On the other hand, the 2'5' oligoadenylate synthetase, present in all vertebrates hitherto assayed, may play regulatory roles other than the activation of a nuclease system by the oligoadenylates.

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We have previously shown (22, 23) that this enzyme is not merely an ATP polymerase and that it can catalyze the synthesis of a series of heterooligonucleotides of a structure ppp A2'(p5'A)$_n$2'p5'N where N stands for rC, rG, rU, T, dC, dG or dA. We have extended this study to other substrates and report the formation of (2'5') (3'5') heteronucleotides, and NAD and tRNA derivatives by the enzyme.

MATERIALS AND METHODS

Chemicals. Radioactive nucleotides were obtained from the Radio Chemical Centre (Amersham, Great Britain), Nucleoside triphosphates from Sigma (St Louis, USA) ; Yeast tRNA from Boehringer (Mannheim, Germany);bovine liver tRNA from Sigma, (St Louis, U.S.A.) ; E. Coli tRNA from C.N.R.S. (Gif s/ Yvette, France) ; Mouse Interferons (S.A., 10^6u/ml) ; rI : rC and rI : rC agarose from Institut Choay, (Montrouge, France). Pure $tRNA^{Val}_{Coli}$ was a gift from J. Vacher of this Institut ; pure Yeast tRNAs were a gift from Dr. G. Dirheimer and his coworkers (Institut de Biologie Moléculaire et Cellulaire, Strasbourg). Cellulose MN 300 from Macherey Nagel (Düren, Germany). Polyethyleneimine (Polymin P) was a gift from BASF France ;

The PEI cellulose thin layer plates were prepared as described by Randerath et al. (24).

Cells growth and interferon treatment. Mouse cells L 929 were grown to confluency in MEM medium containing 6% of new born calf serum and then treated with mouse C 243 interferons (250 units/ml) for 24 hours at 37°C.

Isolation of 2'5' oligoadenylate synthetase. Mouse L cells washed in buffer A : 140 mM NaCl, 35 mM Hepes pH 7,5, 3 mM $MgCl_2$ were lysed in buffer H (25) : 10 mM Hepes pH 7,6, 10 mM KCl, 2 mM $Mg(OAc)_2$, 7 mM 2-mercaptoethanol with 0,5 % NP 40 (2.10^7 cells, in 1ml) for 5 minutes at 0°C. The cell lysate was centrifuged for 6mn in an Eppendorf centrifuge and the enzyme present in the supernatant was then bound to rI : rC agarose beads.

The purification of the enzyme from rabbit reticulocytes was as described previously by Justesen and al. (23).

The concentrations of proteins were determined according to Spector (26).

ASSAY FOR 2'5' NUCLEOTIDYL TRANSFERASE ACTIVITIES

The standard incubation conditions were used as previously described for formation of 2'5' oligoadenylates (22, 23), except that other substrates were added as mentioned in the text, and in some case ATP was excluded. In incubations with NAD^+ the β-form was used.

RESULTS

I. - Formation of heteronucleotides. The rabbit reticulocyte 2'5' oligoadenylate synthetase catalyzes the addition of one unit of NMP to an acceptor yielding 2'5'oligonucleotides (22). The incorporation of NMP is also observed with the mouse L-cell enzyme (Fig. 1a) but with a reduced efficiency compared

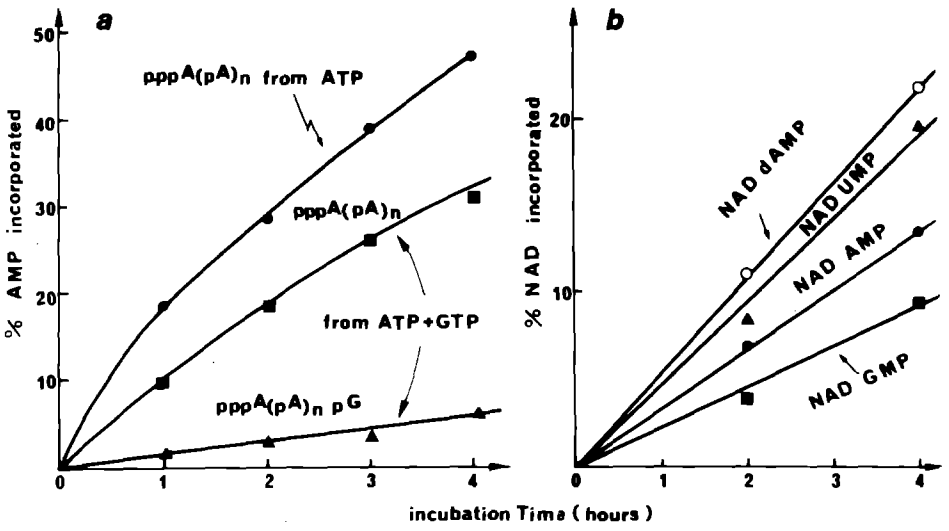


FIGURE 1 : Mouse L-Cell 2'5' oligoadenylate synthetase.

For each incubation 100 µl of an extract of Mouse L-cells treated with interferon (250 units/ml for 24 hours) was added to 15 µl rI : rC agarose. After 15 minutes incubation at room temperature the agarose was washed twice with 500 µl of buffer D. To the pellet of agarose with the oligoadenylate synthetase was added 5 µl of a mixture of : 0.5 mg/ml bovine serum albumin, 0.025 mg/ml rI : rC, and 5 µl of nucleotides in buffer D to give a final concentration in the incubation of :
a) 2 mM ATP, $\alpha(^{32}P)$ ATP (1 µCi) plus 1 mM GTP when present.
b) 1 mM NAD^+ and (^3H) NAD^+ (0.6 µCi) plus another nucleotide as indicated : 2 mM ATP, 1 mM 2'dATP, A mM GTP or 1 mM UTP.
Incubation was at 37°C and at the times indicated 1 µl was withdrawn and spotted onto a PEI-cellulose plate, which was developed in a : 2 M Tris-system and b : the Acetic acid/LiCl system.

to AMP incorporation. The presence of a nucleoside triphosphate other than ATP also reduces the rate of 2'5' oligoadenylate formation indicating a competition between ATP and NTP for the donor site (22). The acceptor can also be a 3'5' oligonucleotide with an AMP group at the 2' (3') terminal end such as 3'5'CpA or 3'5'$(Ap)_3A$ (Table 1).

Nicotinamide adenine dinucleotide (NAD^+) acts as an acceptor for 2' adenylation by the enzyme isolated from interferon treated chicken cells (27). The rabbit reticulocyte enzyme also performs this 2' adenylation and at a rate comparable to the 2'5' oligoadenylate formation (Fig. 2).

Besides accepting AMP, the NAD^+ molecule can accept UMP, CMP, GMP and dAMP and probably other NMP catalyzed by the rabbit reticulocyte synthetase

TABLE 1 : RATE OF FORMATION OF 2'5' OLIGOADENYLATE AND (3'5') (2'5') HETEROOLIGONUCLEOTIDES

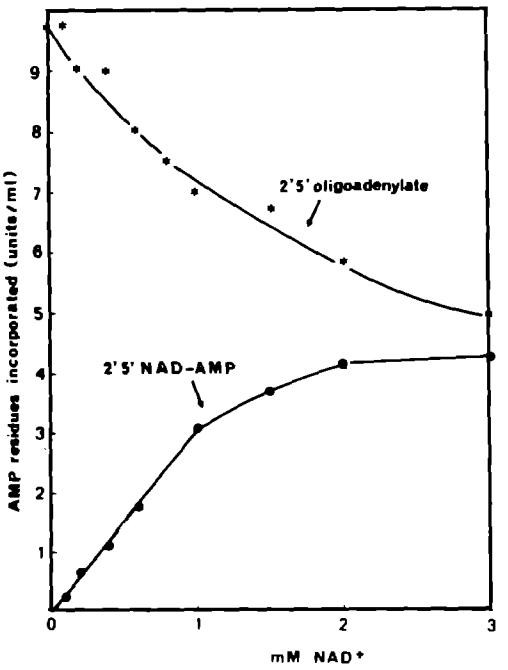
SUBSTRATES :		NMP incorporated nanomoles/min/ml	
R 1mM	2mM	2'5' R - NMP	oligoadenylates 2'5' pppA(pA)n
3'5' CpA	$\alpha[^{32}P]$ ATP	2.5	5.3
3'5' CpA	$[^{3}H]$ UTP	2.0	—
3'5' $(Ap)_3A$	$\alpha[^{32}P]$ ATP	1.3	4.3
—	$\alpha[^{32}P]$ ATP	—	9.7

2'5' oligoadenylate synthetase was incubated under standard condition with the combinations of substrates given in the table at 37°C for 2 hours The reaction was stopped by spotting 5 µl directly onto PEI cellulose plates. The spots corresponding to products and substrates were localized after chromatography in 1 M Acetic acid / 0.3 LiCl-system.
The percent conversion was calculated and used for determination of the enzyme activity.

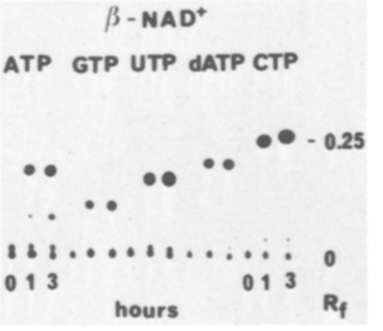
(Fig. 3) and by the mouse L-cell enzyme (Fig. 1b). The formation of NAD-NMP has been demonstrated by radioactive labeling of both NAD^+ and NTP. Furthermore, $NADP^+$ did not serve directly as substrate, but phosphatase treatment to eliminate the 2' phosphate, makes it a substrate as good as NAD^+ (data not shown).

The activities of the different reactions are given in Table 2. UMP, CMP and dAMP as well as AMP are incorporated into NAD at about half the rate of AMP incorporation into 2'5' oligoadenylates. The total activity of AMP incorporation into 2'5' oligoadenylates and into NAD-AMP is more or less constant (Fig. 2) and equal to the activity of 2'5' oligoadenylate formation in the presence of ATP alone. The concomitant formation of NAD-AMP inhibits the rate of formation of oligoadenylate. Furthermore the chromatogram shows that NAD-AMP accepts another AMP group forming NAD-$(pA)_2$.

A third 2'5' oligoadenylate synthetase, isolated from human spleen cells treated with human interferon, also catalyzes the synthesis of hetero-oligonucleotides. (data not shown).



(2)



(3)

FIGURE 2 : Formation of NAD.AMP.

2'5' oligoadenylate synthetase from reticulocyte lysate was incubated with a varying amount of NAD+ and 2 mM ATP and 0.015 µCi $\alpha(^{32}P)$ ATP in a volume of 10 µl at 37°C for 2 hours. 3 µl samples were analyzed by PEI-cellulose chromatography in two system : the 2 M tris system to separate 2'5' oligoadenylates from ATP and the acetic acid/LiCl system to separate ATP and oligonucleotides from NAD-compounds. The activities were calculated from the percentage converted taking into account only linkage formation and not total incorporation.

FIGURE 3 :

Reticulocyte oligoadenylate synthetase was incubated in the standard medium with 1 mM NAD+ and with 1 mM radioactive nucleoside triphosphate (40 µl) : (^{3}H) ATP, (^{14}C) GTP, (^{3}H) UTP, (^{3}H) 2'dATP and (3H) CTP as indicated on the chromatogram. 5 µl of the incubation mixture was spotted onto the PEI cellulose plate at 1 and 3 hours and developed in the Acetic acid/LiCl system.

II. - Adenylation of tRNA. A natural RNA, tRNA, can also be used as a primer for the 2' adenylation by both the rabbit reticulocyte and the mouse L-cell enzyme. Incubations of tRNA and $\alpha(^{32}P)$ ATP with the enzyme give rise to formation of tRNA labelled with (^{32}P) AMP (Fig. 4).

Staining of the tRNA with ethidium bromide or methylene blue results in bands at the same position as the radioactive label. This type of adenylation by 2'5' oligoadenylate synthetase has been obtained with total tRNA

TABLE 2 : COMPARISON OF RATES OF FORMATION OF NAD-NMP DERIVATIVES

SUBSTRATE : 1 mM	1 mM	nanomoles incorporated /min/mg protein
NAD^+	ATP	54.1
NAD^+	GTP	27.7
NAD^+	UTP	56.1
NAD^+	dATP	47.0
NAD^+	CTP	51.6
reference : oligoadenylate formation from ATP alone.		137.5

The present numbers are calculated from the initial rates of incorporation of nucleotides in the incubations shown in figure 3.

from yeast, E. Coli and bovine liver cells and with purified tRNA-species such as $tRNA^{Phe}$, $tRNA^{Val}$ and $tRNA^{Arg}$ from yeast and $tRNA^{Val}$ from E. Coli. The synthesis of 2'5' oligoadenylates still occurs in the presence of tRNA but the rate is reduced. The oligoadenylate synthesis is almost completely inhibited at molar concentration ratios of tRNA : ATP of 1 : 3, whereas the adenylation of tRNA still occurs.

However addition of high concentrations of tRNA (2 mg/ml) does not relieve the absolute dependence on double stranded RNA (rI : rC) for 2' adenylation activity and 2'5' oligoadenylate synthetase activity in an extract of human Wish cells (data not shown). The low activity in the absence of rI : rC in figure 4 is probably due to a contamination of dsRNA in the enzyme preparation from reticulocyte lysate.

DISCUSSION

In this report we have demonstrated that the 2'5' oligoadenylate synthetases isolated from different sources, rabbit reticulocyte lysate, interferon treated mouse L-cells and human spleen cells can use a variety of substrates and catalyze the formation of hetero-oligonucleotides different from the 2'5' oligoadenylates. The substances found to be substrates besides 2'5' oligoadeny-

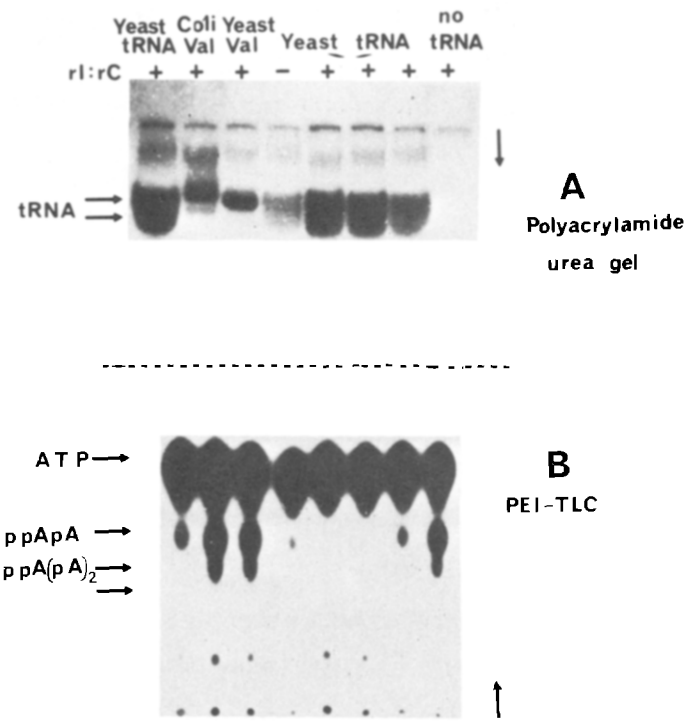


FIGURE 4 :

Adenylation of tRNA and inhibition of the synthesis of 2'5' oligoadenylate by tRNA. The reaction mixture contained in 20 µl : 20 mM Tris-HCl pH 8,0, 25 mM KCl, 1 mM DTT, 10 % glycerol, 0,5 mM $\alpha(^{32}P)$ ATP 0,16 mM yeast tRNA (or E. Coli $tRNA^{Val}$ or $tRNA^{Val}_{Yeast}$ both at 45 µM) 20 µg/ml rI : rC 20 mM Mg $(OAc)_2$, 14 units/ml 2'5' oligoadenylate synthetase. The incubation was at 37°C for 2 hours.
A) The tRNA were separated from other components by electrophoresis on polyacrylamide gel. The gel was prepared similarly as in (28) with the following modifications : 20 % acrylamide, 0.8 % N N' methylene bis-acrylamide and 4 M urea. 10 µl of the incubation mixture was used for each lane. After electrophoresis, the gel was soaked in a 0.001 % solution of ethidium bromide for 20 minutes and photographed under U.V. (picture not shown). The gel was then dried and autoradiographed as usual with a Kodak X-Omat R film.
B) The oligoadenylates synthesized simultaneously with the adenylation of the tRNA were separated on a PEI-cellulose plate as previously described. The picture is a composite representation of these two analysis.

lates and ATP are 3'5' oligonucleotides and NAD^+ as acceptors of 2' adenylation. Nucleoside triphosphates other than ATP have been found to be able to replace ATP as the nucleoside monophosphate donor. Such incorporation of NMP other than AMP to a primer or to a growing nascent oligonucleotide under in vitro assay conditions (22) seems to be a one-unit addition which terminates the chain elongation. One exception observed in the case of 3' deoxyadenosine triphos-

phate that can be polymerized by the enzyme into 2'5' oligomers (R.J. Suhadolnik et al. personal communication).

To be an acceptor a molecule presumably requires a 3' terminal AMP group, as all acceptors contain this. However AMP and other nucleoside monophosphates did not serve as acceptors (data not shown). The AMP group has to be linked to a group (R) at its 5' end to be active : RpA where R stands for nicotinamide ribose phosphate (forming NAD^+), cytidine (forming 3'5' CpA), pyrophosphate (forming ATP) or a polynucleotide (like tRNA).

The biological activities of some of these 2'5' hetero-oligonucleotides have been investigated in human fibroblast cells. The series of 2'5' pppApApN, where N = rC, rG, rU, dT, dC, dG and dA, has been assayed for the ability to inhibit in vivo protein synthesis compared to the 2'5' pppApApA, which is the normal inhibitor. The oligonucleotides were introduced into the cells by the method of Ca^{++} precipitation under conditions similar to those described by Hovanessian et al. (16). Only the deoxyadenosine derivative showed inhibition, but all heteronucleotides had a competitive effect on the inhibition by 2'5' oligoadenylate (29). On the other hand NAD-NMP has no inhibitory effect on the synthesis of protein in vivo using the same cell system.

Ball has reported (27) that the 2'5' NAD-AMP cannot be reduced like NAD^+ to NADH. We are currently investigating the role of NAD-NMP in metabolic pathways controlled by NAD^+ or $NADP^+$. Whatever the function of 2'5' NAD-NMP is, the rate of formation of this molecule is comparable to that of 2'5' oligoadenylates at the same substrate concentrations. Moreover, under these conditions, the presence of NAD^+ inhibits the synthesis of 2'5' oligoadenylates from ATP.

The adenylated tRNAs are particularly interesting as they are the first natural nucleic acid containing 2'5' as well as 3'5' phosphodiester linkages. We are investigating whether tRNApA has any effect on protein synthesis or on the viral transcription using tRNA as a primer. As in the case of NAD^+, tRNA also inhibits the synthesis of 2'5' oligoadenylate from ATP.

Two suggestions can be drawn from this study. First NAD^+ and tRNA could play a regulator role for the synthesis of 2'5' oligoadenylates. This adds

another feature to the regulation model proposed by Revel and co-workers (30) that the 2'5' oligoadenylate level would be controlled by the balance between synthesis and degradation. Second, the adenylated tRNA and the NAD-NMP derivatives could be important compounds involved in the control mechanisms of cell functions, mediated by interferon or by other agents that induce the 2'5' oligoadenylate synthetase.

Finally, due to the multiple reactions catalyzed by this enzyme, we suggest that the enzyme should be called 2'5' nucleotidyl-transferase.

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REFERENCES

1. Kerr, I.M. and Brown R.E. (1978) Proc. Nat. Acad. Sci. USA 75, 256-260
2. Hovanessian, A.G. and Kerr, I.M. (1978) Eur. J. Biochem. 84, 149-159
3. Stark, G.R. Dower, W.J., Schimke, R.J., Brown, R.E. and Kerr, I.M. (1979) Nature (London) 278, 471-473
4. Shimizu, N. and Sokawa, Y. (1979) J. Biol. Chem. 254, 12034-12037
5. Zilberstein, A., Kimchi, A., Schmidt, A. and Revel, M. (1978) Proc. Nat. Acad. Sci. USA 75, 4734-4738
6. Farrell, P.J., Sen, G.C., Dubois, M.F., Ratner, L., Slattery, E. and Lengyel, P. (1978) Proc. Nat. Acad. Sci. USA 75, 5893-5897
7. Minks, M.A., Benvin, S., Maroney, P.A. and Baglioni, C. (1979) J. Biol. Chem. 254, 5058-5064
8. Ball, A.L. (1979) Virology 94, 282-296
9. Baglioni, C. Minks, M.A. and Maroney, P.A. (1978) Nature (London) 273, 684-687
10. Ball, L.A. and White, C.N. (1979) Virology 93, 348-356
11. Ratner, L., Wiegand, R.C. Farrell, P.J., Sen, G.C., Cabrer, B. and Lengyel P. (1978) Biochem. Biophys. Res. Commun. 81, 947-954
12. Vaquero, C.M. and Clemens, M.J. (1979) Eur. J. Biochem. 98, 245-252
13. Williams, B.R.G., Gilbert, C.S. and Kerr, I.M. (1979) Nucleic Acid Res. 6, 1335-1350
14. Schmidt, A., Zilberstein, A. Shulman, L., Federman, P. Berissi, H. and Revel, M. (1978) FEBS Letters 95, 257-264
15. Minks, M.A. Benvin, S., Maroney, P.A. and Baglioni, C. (1979) Nucleic Acids Res. 6 767-780
16. Hovanessian, A.G. Wood, I., Meurs, E. et Montagnier, L. (1979) Proc. Nat. Acad. Sci. USA 76, 3261-3265
17. Williams, B.R.G., Golgher, R.R. and Kerr, I.M. (1979) FEBS Letters 105, 47-52

18.Hovanessian, A.G. and Wood, J.N. (1980) Virology 101, 81-90
19.Wood, J.N., and Hovanessian, A.G. (1979) Nature (London) 282, 74-76
20.Verhaegen, M., Divizia, M., Vandenbusshe, P., Kuwata, T. and Content, J. (1980) Proc. Nat. Acad. Sci. USA 77, 4479-4483
21.Williams, B.R.G., Golgher, R.R., Brown, R.E., Gilbert, C.S. and Kerr, I.M. (1979) Nature 282, 582-586
22.Justesen, J. Ferbus, D., Thang, M.N. (1980) Proc. Nat. Acad. Sci. USA 77, 4618-4622
23.Justesen, J. Ferbus, D., Thang, M.N. (1980) Nucleic Acid Res. 8, 3073-3085
24.Randerath, E. and Randerath, K. (1967) J. Chromatog. 31, 485-499
25.Hovanessian, A.G., La Bonnadière, C. and Falcoff, E. (1980) J. Interferon Res. 1, 125-135
26.Spector, T. (1978) Anal. Biochem. 86, 142-146
27.Ball, L.A. and White, C.N. (1979) In Regulation of Macromolecular Synthesis by Low molecular weight mediators eds Koch, H. and Richter, D. (Academic Press, New York) pp 303-317
28.Ikemura, T. and Dahlberg, J.E. (1973) J. Biol. Chem. 248, 5024-5032
29.Drocourt , J.L., Dieffenbach, C.N., T'so P.O.P., Justesen, J., and Thang, M.N. (submitted)
30.Schmidt, A., Chernajowsky, Y., Shulman, L., Federman, P., Berissi, H. and Revel, M. (1979) Proc. Nat. Acad. Sci. USA, 76, 4788-4792.