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ENZYMATIC SYNTHESIS OF THE 2',5'-A_4 TETRAMER ANALOG, 2',5'-ppp3'dA(p3'dA)$_3$, BY RABBIT RETICULOCYTE LYSATES: BINDING AND ACTIVATION OF THE 2',5'-A_n DEPENDENT NUCLEASE, HYDROLYSIS OF mRNA, AND INHIBITION OF PROTEIN SYNTHESIS

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SUMMARY: The structurally modified 2',5'-pppA(pA)$_3$ (tetramer) analog, 2',5'-ppp3'dA(p3'dA)$_3$ (referred to as $p_3$3'dA$_4$), synthesized by 2',5'-A_n synthetase from cordycepin 5'-triphosphate (3'dATP) in lysates from rabbit reticulocytes has the same structure as chemically synthesized $p_3$3'dA$_4$. Under our assay conditions, when the ^{14}C-amino acid concentration is $\geq$50 µM, $p_3$3'dA$_4$ (enzymatically or chemically synthesized) is the most potent inhibitor of protein synthesis in lysates from rabbit reticulocytes reported to date (61% inhibition at 6.7 x 10^{-10} M). $p_3$3'dA$_4$ binds to and activates the 2',5'-A_n dependent nuclease to hydrolyze VSV [^{3}H]mRNA. The 3'-hydroxyl groups of the adenylate of the p_3A_4 are not required to activate the 2',5'-A_n dependent nuclease in lysates from rabbit reticulocytes.

2',5'-A_n synthetase, found in lysates from rabbit reticulocytes and interferon treated cells, synthesizes a series of 2',5'-A_n with certain dsRNAs. 2',5'-A_n complexes with the 2',5'-A_n dependent nuclease which then hydrolyzes RNA (1). Because 2',5'-A_n is rapidly hydrolyzed by 2',5'-phosphodiesterase, 2',5'-A_n analogs with extended half-lives are needed to study the function of 2',5'-A_n in the cell. Hence, we first reported that enzymatically synthesized $p_3$3'dA$_n$ inhibited protein synthesis in lysates and was more resistant to hydrolysis by 2',5'-phosphodiesterase than the naturally occurring p_3A_n (2-4). We subsequently reported that the 5'-dephosphorylated "core" 3'dA$_3$ inhibited the transformation of Epstein-Barr virus infected human lymphocytes (5) and inhibited the formation of EBV-associated nuclear antigen (EBNA)(6). Because the naturally occurring p_3A_3 (trimer) is not a potent

Abbreviations used are: 2',5'-A_n, oligomers of adenylic acid with 2',5'-phosphodiester linkages and a 5'-triphosphate; core, 5'-dephosphorylated 2',5'-A_n; p_3A_4, adenylic acid tetramer; $p_3$3'dA$_4$, 3'-deoxyadenylic acid tetramer; BAP, bacterial alkaline phosphatase; SVPD, snake venom phosphodiesterase.

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inhibitor of protein synthesis in lysates, whereas p_3A_4 (tetramer) is a potent inhibitor but is rapidly hydrolyzed, we have enzymatically synthesized $p_3 3'dA_4$ (tetramer) which inhibits protein synthesis and binds to and activates the 2',5'-A_n dependent nuclease to hydrolyze mRNA.

MATERIALS AND METHODS

[^{3}H]3'dATP (14.6 Ci/mmol), p_3A_4[^{32}P]pCp (3000 Ci/mmol), [U-^{14}C]leucine (330 mCi/mmol) and [U-^{14}C]valine (275 mCi/mmol) were from Amersham; p_3A_3 and core A_3 and A_4 from P.L. Biochemicals. 3'dATP (7) was determined to be free of ATP by HPLC. Chemically synthesized core $3'dA_3$ and $3'dA_4$ were supplied by Dr. W. Pfleiderer, Konstanz University. Chemically synthesized $p_3 3'dA_3$ and $p_3 3'dA_4$ were generous gifts from Dr. P. Torrence, NIH. Thin layer chromatography was performed using Eastman Chromagram cellulose (No. 13254), Brinkman PEI-cellulose, and Brinkman DEAE-cellulose. Solvents: A: isobutyric acid/ammonium hydroxide/water, 66:1:33, v/v/v; B: 0.75 M KH_2PO_4, pH 3.5; C: 0.1 M ammonium formate / 9 M urea / 1 mM Na_2-EDTA, after a short pre-run in water (8). $p_3 3'dA_4$ was synthesized and isolated from lysates (3,4) with the following modifications: the KCl concentration of the column wash buffer was 25 mM; incubations were at 37°C, 5 hr, with [^{3}H]3'dATP (200 μCi). Structural elucidation of the [^{3}H]$p_3 3'dA_4$ was accomplished by enzymatic digestion with BAP, SVPD, T2 RNase, and alkaline hydrolysis (3,4,9). Lysates were from: injection of rabbits with phenylhydrazine (10), Clinical Convenience, or Dr. P. Torrence. Inhibition of protein synthesis was determined as reported from this laboratory (11). The concentration of the enzymatically synthesized $p_3 3'dA_4$ was determined by radiobinding assay (12) using chemically synthesized $p_3 3'dA_4$ (250 μM) supplied by Dr. Torrence as the standard.

The 2',5'-A_n dependent nuclease was partially purified from lysates as follows. Lysates (200 ml) were centrifuged (100,000 x g, 4 hr, 0°C). Postribosomal supernatant (100 ml) was passed through a DEAE-cellulose column (2.5 x 25 cm) equilibrated with buffer A (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA and 5% glycerol). The column was washed with 500 ml of buffer A and the proteins retained on the column were displaced with buffer A supplemented with 300 mM KCl. Fractions were collected and assayed for nuclease activity (hydrolysis of VSV [^{3}H]mRNA) by oligo(dT)-cellulose column chromatography in a 2 hr incubation (13). A sharp peak of nuclease activity (tubes 5 and 6) had only 5% of the original 2',5'-phosphodiesterase activity.

RESULTS AND DISCUSSION

Enzymatic synthesis and isolation of [^{3}H]$p_3 3'dA_4$. [^{3}H]$p_3 3'dA_4$ was displaced from the DEAE-cellulose column with chemically synthesized $p_3 3'dA_4$ (charge -7) (Fig. 1a). Another aliquot of [^{3}H]$p_3 3'dA_4$ was isolated without the addition of $p_3 3'dA_4$ marker (Fig. 1b) for proof of structure and biological studies.

Proof of structure of $p_3 3'dA_4$. Five methods were used to prove the structure and purity of the putative [^{3}H]$p_3 3'dA_4$. First, all [^{3}H]$p_3 3'dA_4$ (charge -7) resided in the 5'-triphosphate region (R_f 0.30, PEI-cellulose, solvent B). Second, the [^{3}H]$p_3 3'dA_4$ was treated with alkali to demonstrate the absence of adenylate residues. There was no change in the inhibition of protein synthesis by the enzymatically syn-

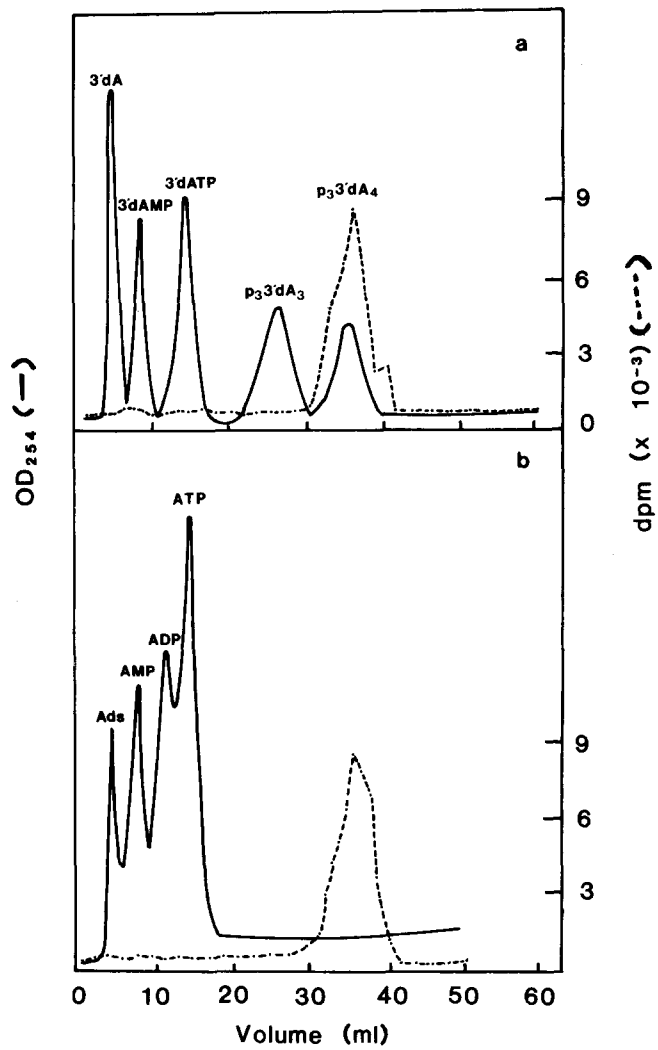

Figure 1. DEAE-cellulose column chromatography of [^{3}H]$p_3$3'dA$_4$ from incubations with [^{3}H]3'dATP. The second one-ml fraction displaced from DEAE cellulose columns with 350 mM KCl was dialyzed, adjusted to 50 mM NaCl and fractionated on a DEAE-cellulose column (3,4). Panel A: 56,000 dpm [^{3}H]$p_3$3'dA$_n$; panel B: 47,000 dpm [^{3}H]$p_3$3'dA$_n$. UV markers are indicated. Fractions in panels A and B corresponding to [^{3}H]$p_3$3'dA$_4$ were pooled, dialyzed and lyophilized.

thesized $p_3$3'dA$_4$ before and after alkaline hydrolysis (Fig. 2, ◇-◇). The same results were observed with the chemically synthesized $p_3$3'dA$_4$ (□-□). As previously reported (9), p_3A_4 (Δ-Δ) was completely hydrolyzed by alkali (▲-▲). Third, only [^{3}H]3'dAMP (R_f 0.73) was isolated following SVPD hydrolysis and cellulose tlc (solvent A) of the enzymatically synthesized [^{3}H]$p_3$3'dA$_4$. Fourth, all of the ^{3}H following BAP hydrolysis of [^{3}H]$p_3$3'dA$_4$ was in the core 3'dA$_4$ (R_f 0.82); there was no ^{3}H in core A$_4$ (R_f 0.66)(R_f values of control nucleosides and nucleotides are as

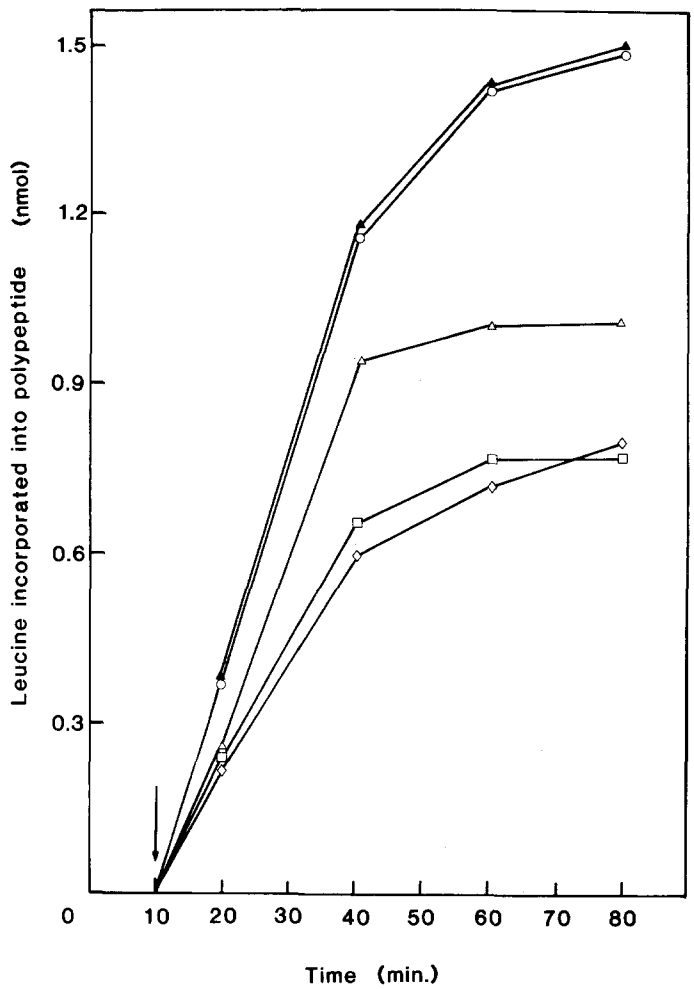

Figure 2. Inhibition of protein synthesis in lysates by 10 nM p_3A_4 and $p_33'dA_4$ before and after alkaline hydrolysis. Conditions: control, o-o; p_3A_4, Δ-Δ; chemically synthesized $p_33'dA_4$, □-□; enzymatically synthesized $p_33'dA_4$, ◇-◇; aliquot removed at time zero of 0.3 N KOH hydrolysis (p_3A_4, Δ-Δ; $p_33'dA_4$, □-□; enzymatically synthesized $p_33'dA_4$, ◇-◇); aliquot removed following 0.3 N KOH hydrolysis, 18 hr, 37°C (p_3A_4, ▲-▲; $p_33'dA_4$, □-□; enzymatically synthesized $p_33'dA_4$, ◇-◇). 0.3 N KOH hydrolysis of [U-^{14}C]ATP, 18 hr, 37°C and PEI-cellulose tlc (solvent B) showed all of the ^{14}C in the ATP region. Each symbol is an average of five experiments in duplicate, standard error ≤ 3%. Arrow indicates addition of master mix. All samples were dialyzed 45 min, 4 l. H_2O, 0°C. Assays: 30 µl; [U-^{14}C]leucine: 63 µM; dpm added: 275,000; final specific activity: 146,000 dpm/nmole.

reported)(4-6). Fifth, DEAE-cellulose tlc (net charge separation)(8) separated core $3'dA_3$, $3'dA_4$, and $3'dA_5$ (charges -2,-3,-4)(solvent C); all of the ^{3}H following BAP hydrolysis of [^{3}H]$p_33'dA_4$ resided in the region equivalent to chemically synthesized core $3'dA_4$ (R_f 0.23, charge -3). The absence of ^{3}H in the pentamer core region (R_f 0.10, charge -4) indicated that $p_23'dA_5$ (charge -7) was not displaced with $p_33'dA_4$ from the DEAE-cellulose column.

TABLE 1: INHIBITION OF PROTEIN SYNTHESIS BY p_3A_4 AND $p_3 3'dA_4$ IN LYSATES FROM RABBIT RETICULOCYTES[a]

Lysate:	I[b]		II[c]	III[d]	
Experiment:	1	2	3	4	5
Amino acid added:	[U-^{14}C]leucine			[U-^{14}C]valine	
Final conc. (μM):	10	90	90	16	50
Specific activity (dpm/pmole):	760	83	83	550	178
pmoles ^{14}C added:	300	300	300	1820	1820
pmoles unlabeled amino acid added:	0	2400	2400	0	3750
total pmoles added:	300	2700	2700	1820	5570
	pmoles incorporated into polypeptide				
control[e] (no addition)	270	2090	970	1460	2050
p_3A_4					
6.7×10^{-9} M	250	850	270	1280	1060
6.7×10^{-10} M	270	1640	410	1460	2010
6.7×10^{-11} M	270	1910	980	1400	2180
$p_3 3'dA_4$					
6.7×10^{-9} M	250	1040	300	1390	1160
6.7×10^{-10} M	260	1790	380	1530	1880
6.7×10^{-11} M	260	2020	860	1550	2140

[a]Lysates preincubated with p_3A_4 or $p_3 3'dA_4$ at 30°C, 10 min followed by addition of master mix and an additional 60 min incubation. Expt. 1-3 according to Lennon et al. (11): 30 μl assay; [U-^{14}C]leucine added: 220,000 dpm. Expt. 4,5 according to Safer et al. (14): 110 μl assay; [U-^{14}C]valine added: 1×10^6 dpm.

[b]Lysate from Clinical Convenience.

[c]Lysate from phenylhydrazine injection of rabbits (15).

[d]Lysate, master mix, [U-^{14}C]valine, and $p_3 3'dA_4$ from Dr. P. Torrence.

[e]dpm incorporated into polypeptide/5 μl aliquot, expt. 1-5: 34,200, 27,870, 12,930, 36,500 and 16,770, respectively.

Inhibition of protein synthesis by p_3A_4 and $p_3 3'dA_4$. We have compared the inhibition of protein synthesis by p_3A_4 and $p_3 3'dA_4$ with three different lysate preparations, two different assay mixtures, and various concentrations of ^{14}C-leucine and valine. Two observations emerge from these studies (Table 1). First, a

fixed concentration of p_3A_4 or $p_33'dA_4$ gives varying degrees of inhibition with different lysates. For example, at 6.7×10^{-10} M $p_33'dA_4$, there was a 16% inhibition with lysate I (expt. 2) and a 61% inhibition with lysate II (expt. 3). Similar results were obtained with p_3A_4. Second, inhibition of protein synthesis by p_3A_4 or $p_33'dA_4$ is critically dependent on the concentration of amino acids. When the concentration of one amino acid is rate limiting such that translation becomes regulated by the rate of elongation, the inhibitory effects of p_3A_4 and $p_33'dA_4$ on protein synthesis become marginal at best (Table 1). For example, when ^{14}C-leucine is 10 μM or ^{14}C-valine is 16 μM, there is little or no inhibition of protein synthesis by p_3A_4 or $p_33'dA_4$, regardless of lysate or assay mixture (expt. 1,4); however, when the concentration of ^{14}C-leucine is increased to 90 μM (expt. 2,3) or the ^{14}C-valine is increased to 50 μM (expt. 5), 6.7×10^{-9} M p_3A_4 and $p_33'dA_4$ are potent inhibitors of protein synthesis. With 90 μM ^{14}C-leucine or 50 μM ^{14}C-valine, $p_33'dA_4$ is a better inhibitor of protein synthesis than is p_3A_4 at 6.7×10^{-10} M (expt. 3,5). Neither p_3A_4 nor $p_33'dA_4$ had any effect on the energy regenerating system as measured by the conversion of AMP to ATP. Omission of exogenous ATP/GTP did not affect the inhibition of protein synthesis by p_3A_4 and $p_33'dA_4$ (data not shown). As was seen with inhibition of protein synthesis by the tetramers, high concentrations of amino acids are essential in order to see inhibition of protein synthesis by p_3A_3 and $p_33'dA_3$ (trimers)(manuscript in preparation). p_3A_3, $p_33'dA_3$, p_3A_4, and $p_33'dA_4$ (10^{-8} M) also inhibit protein synthesis in L929 cells as determined by calcium phosphate coprecipitation (manuscript in preparation).

Activation of the 2',5'-A_n dependent nuclease and hydrolysis of VSV [^{3}H]mRNA. When the partially purified 2',5'-A_n dependent nuclease was incubated with 10^{-8} to 10^{-11} M p_3A_4 or $p_33'dA_4$, there was more degradation of VSV [^{3}H]mRNA by $p_33'dA_4$ (Fig. 3a). In addition, $p_33'dA_4$ competes off p_3A_4[^{32}P]pCp better than p_3A_4 (Fig. 3b). In conclusion, the results reported here show that the 3'-hydroxyl groups of p_3A_n are not required for activation of the 2',5'-A_n dependent nuclease and subsequent inhibition of protein synthesis.

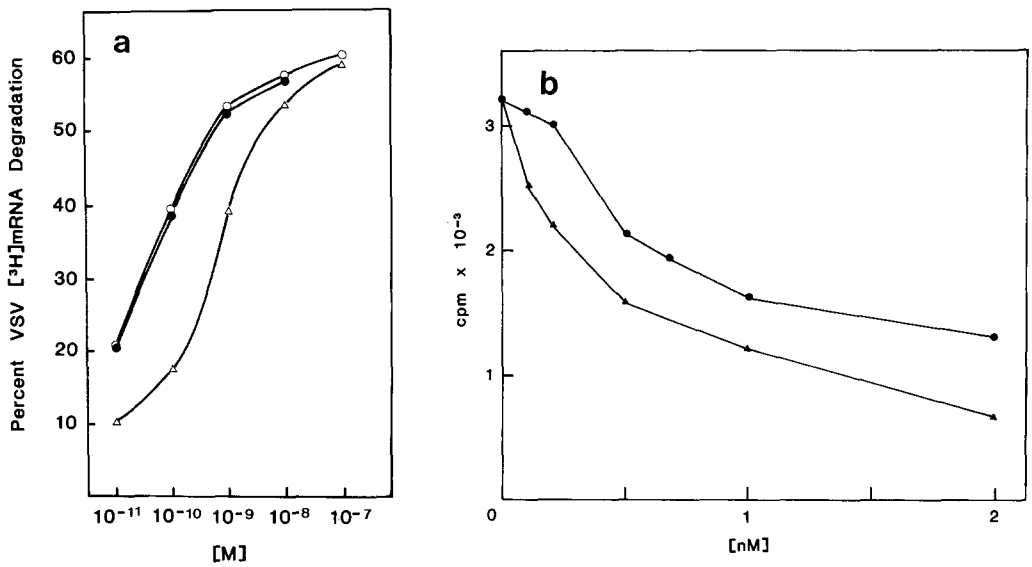

Figure 3a. VSV [^{3}H]mRNA hydrolysis by p_3A_4 and $p_3 3'dA_4$. Assays were done as described (13). 60,000 dpm (0.1 μg) VSV [^{3}H]mRNA added per assay. VSV [^{3}H]mRNA was isolated from Vero cells according to Weber et al. (15). Chemically synthesized $p_3 3'dA_4$, o-o; enzymatically synthesized $p_3 3'dA_4$, ▲-▲; p_3A_4, ●-●. In control assays (nuclease with no addition), 80% of the VSV [^{3}H]mRNA was recovered. Data points are an average of three experiments, standard error ≤ 2.5%. b. Radiobinding assays of p_3A_4 and $p_3 3'dA_4$. Chemically or enzymatically synthesized $p_3 3'dA_4$, ▲-▲; p_3A_4, ●-●. Data points are an average of five experiments, standard error ≤ 2.5%.

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