Activation of Ribonuclease L by (2′–5′)$(A)_4$–Poly(L-lysine) Conjugates in Intact Cells†

Bernard Bayard,* Catherine Bisbal, and Bernard Lebleu

Laboratoire de Biochimie des Protéines, UA CNRS 1191, Université de Montpellier II, 34060 Montpellier Cedex, France

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ABSTRACT: Molecular hybrids were synthesized by coupling (2′–5′)$(A)_n$ oligoadenylates or 2–5A, an intracellular mediator involved in antiviral activity of interferons (IFNs), with poly(L-lysine) used as a membrane carrier. (2′–5′)$(A)_n$ in its free form was not taken up by cells, probably because of its ionic character. Conjugation with the polypeptide carrier overcame this problem and enabled its pharmacological properties to be developed. The α-glycol group of individual (2′–5′)$(A)_n$ oligomers was oxidized by periodate oxidation and conjugated by an amino reductive reaction to poly(L-lysine), M_r 14 000, in a molar ratio of 5:1. These hybrid molecules left the biologically active 5′ end moiety of the (2′–5′)$(A)_n$ molecule unchanged, and in particular its triphosphate group, and stabilized the molecule by increasing its resistance to phosphodiesterase hydrolysis. A dose-dependent inhibition of virus growth was observed on concomitant incubation of (2′–5′)$(A)_n$–poly(L-lysine) conjugates with vesicular stomatitis virus infected L1210 cell cultures. This was a result of the activation of the (2′–5′)$(A)_n$-dependent endoribonuclease (RNase L) by intracellularly delivered (2′–5′)$(A)_n$ as in some IFN-treated virus-infected cells. Indeed, (2′–5′)$(A)_n$–poly(L-lysine) conjugates bind RNase L effectively as can be seen from their ability to compete with authentic (2′–5′)$(A)_n$ in a cell-free radiobinding assay. Moreover, (2′–5′)$(A)_n$–poly(L-lysine) conjugates promote transient inhibition of protein synthesis and a characteristic cleavage pattern of ribosomal RNAs in intact cells. Poly(L-lysine) thus represents an effective vector for the delivery of (2′–5′)$(A)_n$ or its analogues to intact cells and is thus a potentially interesting tool for the study of the biological role of (2′–5′)$(A)_n$ and its possible utilization in antiviral and antineoplasic chemotherapy.

Evidence of a double-stranded RNA (dsRNA) and ATP-activated endoribonuclease (Brown et al., 1976) and of a low molecular weight inhibitor of protein synthesis (Roberts et al., 1976) in extracts from interferon- (IFN) treated cells led to the elucidation of the (2′–5′)$(A)_n$ pathway (Kerr & Brown, 1978). In short, interferon-induced (2′–5′)$(A)_n$ synthetase activity polymerizes ATP into (2′–5′)$(A)_n$ or 2–5A, a series of 2′–5′-linked adenylic oligoribonucleotides, on activation by synthetic [i.e., poly(rI)·poly(rC)] or natural (i.e., viral replicative forms) dsRNA structures. (2′–5′)$(A)_n$ itself binds with high affinity and, hence, activates an endoribonuclease (RNase L or F) that is found in most vertebrate cells and tissues [Johnston & Torrence (1984) for a review].

Initially discovered as a mediator of IFN-induced antiviral activity, the (2′–5′)$(A)_n$ system may have wider significance. Indeed, the level of (2′–5′)$(A)_n$ synthetase varies considerably in different cell lines and tissues in function of growth rate, differentiation stage, and hormonal responses [Stark et al. (1979) and Silverman (1984) for a review]. In regenerating rat liver for instance, both (2′–5′)$(A)_n$ synthetase activity and the (2′–5′)$(A)_n$ intracellular level vary in inverse relation to mitogenic activity (Etienne-Smekens et al., 1983). Functional (2′–5′)$(A)_n$ and (2′–5′)$(A)_n$-related material accumulate in growth-arrested T98G neuroblastoma cells (Reid et al., 1984). These, together with a steadily increasing number of other examples suggest that (2′–5′)$(A)_n$ and (2′–5′)$(A)_n$-related substances may be involved in the control of cell growth and differentiation, as well as in certain cellular responses to hormones. Finally, all the components of the (2′–5′)$(A)_n$ system have been observed in cell nuclei (Nilsen et al., 1982; St Laurent et al., 1983). Seen together with the occurrence of dsRNA regions capable of activating (2′–5′)$(A)_n$ synthetase in large molecular weight nuclear RNA (Hn RNA) and the recent discovery of 2′–5′ phosphodiester linkages in nuclear RNA [Keller (1984) for a review], this suggests possible involvement of the (2′–5′)$(A)_n$ system in the maturation of eukaryotic mRNAs.

However, experimental evidence of a role of (2′–5′)$(A)_n$ and related substances at these levels is essentially circumstantial. Likewise, the precise role played by the (2′–5′)$(A)_n$ system as a mediator of IFN-induced antiviral activity remains to be determined since IFNs induce several other enzymatic activities as well as additional proteins of unknown function [Johnston & Torrence (1984) for a review].

It would thus be of great value to be able to activate or shut off selectively the functioning of the (2′–5′)$(A)_n$ system in intact cells or tissues in order to evaluate its biological role as well as its possible utilization in antiviral and antineoplasic chemotherapy. Since the search for the appropriate mutants has not yet been successful [Lebleu & Content (1982) for a review], tools to introduce agonists or antagonists of the (2′–5′)$(A)_n$ pathway represent an interesting and potentially useful alternative.

The introduction of (2′–5′)$(A)_n$ or (2′–5′)$(A)_n$ analogues in intact cultured cells by calcium phosphate coprecipitation [first carried out by Hovanessian et al. (1979)], by permeabilization in hypertonic medium (Williams & Kerr, 1978), or by microinjection with micropipets (Higashi & Sokawa, 1982; Bayard et al., 1984) leads to transient inhibition of several biological parameters including cellular and viral protein synthesis and multiplication of viruses. In the same way, the introduction of a stable analogue inhibitor of RNase L by the

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calcium phosphate technique was recently used as a tool to assess the role of the $(2'-5')(A)_n$ system in the antiviral activity of IFNs on picornaviruses (Watling et al., 1985).

Although successful for the introduction of $(2'-5')(A)_n$ or $(2'-5')(A)_n$ derivatives in intact cultured cells, these techniques are relatively drastic and disturb cell metabolism at least temporarily. Furthermore, they cannot be extrapolated to the in vivo experimental work, which will be required both for study of the role of $(2'-5')(A)_n$ and for its possible use in chemotherapy.

Less polar dephosphorylated or "core" $(2'-5')(A)_n$ derivatives do promote an antimitogenic activity (Kimchi et al., 1981) initially thought to be associated with cell penetration and intracellular rephosphorylation. Although core $(2'-5')(A)_n$ or the products of its degradation might have an antimitogenic activity, no direct evidence has been obtained for the involvement of RNase L in their action since, for example, the pattern of ribosomal RNA cleavage characteristic of RNase L activation has not been observed in cells treated with authentic core $(2'-5')(A)_n$ (Eppstein et al., 1983) and no antiviral activity has been observed with microinjected core $(2'-5')(A)_n$ or stabilized core $(2'-5')(A)_n$ derivatives (Bayard et al., 1984). Analogues of $(2'-5')(A)_n$ have been encapsulated in the aqueous compartment of protein A coated liposomes whose interaction, in the presence of the appropriate monoclonal antibodies, with cells bearing cognate surface antigens leads to an antiviral activity (Bayard et al., 1985a,b). However, further use might be limited by the amount of $(2'-5')(A)_n$ delivered to cells and by the in vivo stability and clearance problems usually associated with this technology. Interesting attempts to decrease the hydrophylic character of $(2'-5')(A)_n$ to ease their passage through the plasma membrane while maintaining their biological activity are now being made by several groups. For example, 5'-capped derivatives of $(2'-5')(A)_n$ that are stable in serum but rapidly cleaved into $(2'-5')(A)_n$ in cell-free extracts have been synthesized (Imai & Torrence, 1984), but their activity in intact cells has not yet been demonstrated.

We used poly(L-lysine) conjugates to introduce $(2'-5')(A)_n$ into cultured murine leukemic L1210 cells and describe their biological activity and the mechanism of their action. Poly(L-lysine) has been described as a versatile macromolecular carrier enabling the efficient intracellular transport of somewhat different molecules such as proteins (Shen & Ryser, 1978) or low molecular weight drugs (e.g., methotrexate in resistant CHO cells, Ryser & Shen, 1978) that do not normally penetrate cells to a significant extent.

Experimental Procedures

Materials. Media for cell cultures were obtained from Eurobio (Paris) and sera from Flow Laboratories. Poly(rI)·poly(rC) was obtained from P-L Biochemicals and bacterial alkaline phosphatase (EC 3.1.3.1) III-R from Sigma. Adenosine triphosphate was supplied by Merck, and DEAE-Trisacryl M was produced by l'Industrie Biologique Française. Poly(L-lysine) of M_r 14000 was from Sigma. L-[^{35}S]-Methionine (sp act. 1109 Ci/mmol) was purchased from New England Nuclear. The TSK 545 DEAE column for HPLC was supplied by LKB. ^{32}P-Labeled (2'-5')ApCp with a sp act. of 3000 Ci/mmol was from Amersham.

Cell Cultures and Viruses. L929 cells were grown in minimal essential medium supplemented by 5% (v/v) donor horse serum, 3 g/L bactotryptose phosphate broth, 3.4 g/L glucose, 60 IU/mL penicillin, and 50 μg/mL streptomycin. L1210 cells were maintained in RPMI 1640 medium supplemented by 5% (v/v) fetal calf serum and antibiotics as above. The Indiana strain of vesicular stomatitis virus (VSV) was grown in L929 cell monolayers and titrated by plaque assay.

Enzymatic Synthesis, Isolation, and Characterization of $(2'-5')(A)_n$. The $(2'-5')(A)_n$ oligonucleotides were synthesized enzymatically with 2'-5' oligoadenylate synthetase (EC 2.7.7) from HeLa cell extracts by the procedure of Minks et al. (1979). Cytoplasmic extracts from HeLa cells treated with 200 units/mL murine IFN α/β (a gift of Dr. I. Gresser, IRCSC Villejuif) were incubated with 5 mM ATP and 20 μg/mL poly(rI)·poly(rC) for 2 h, boiled for 3 min at 100 °C, and centrifuged at 10000*g* for 10 min. Milligram amounts of oligomers were separated by HPLC on a TSK-DEAE column that was eluted with a linear gradient of 125–500 mM triethylammonium bicarbonate buffer (TEAB), pH 8.5. Fractions containing individual oligomers were pooled, concentrated in vacuo, and coevaporated 3 times with water in order to remove TEAB. Chain lengths of purified oligomers were characterized by HPLC on a μBondapak C_{18} column in ammonium phosphate buffer according to Brown et al. (1981).

Conjugation of $(2'-5')(A)_4$ *Oligonucleotides and Poly(L-lysine).* A 4-μL aliquot of sodium metaperiodate (0.6 μmol in 0.1 M sodium acetate buffer, pH 4.75) was added to an ice-cold solution of $(2'-5')(A)_4$ (25 OD_{260}, 0.6 μmol) in 400 μL of distilled water. The reaction mixture was stirred on ice for 30 min; 400 μL of poly(L-lysine) (0.14 μmol in 0.2 M phosphate buffer, pH 8.0) and 200 μL of sodium cyanoborohydride (20 μmol in 0.2 M phosphate buffer, pH 8.0) were added. The mixture was incubated for 2 h at room temperature and then loaded on a Sephadex G-50 column equilibrated with 0.1 M sodium acetate buffer, pH 4.75. Each fraction was assayed for its $(2'-5')(A)_n$-poly(L-lysine) content by the method described by Lowry et al. (1951) and by absorbance at 260 nm.

Chemical Modification of $(2'-5')(A)_4$. Pure $(2'-5')(A)_4$ (1 μmol) was modified at the 2'-terminal ribose residue in order to increase its stability with regard to phosphodiesterase degradation. In some experiments, this stabilized $(2'-5')(A)_4$ was used as control in the presence of free poly(L-lysine). Periodate oxidation of $(2'-5')(A)_4$ was carried out as described above with an equimolar amount of sodium metaperiodate at 4 °C for 30 min and reduced at 4 °C for 1 h by 100 μL of 0.1 M sodium borohydride in 0.1 M borate buffer, pH 9.0. The purity of this modified oligomer was assessed by HPLC (Brown et al., 1981).

Radiobinding Assay for $(2'-5')(A)_4$ *and* $(2'-5')(A)_4$-*Poly(L-lysine) Conjugates.* Radiobinding assays were performed according to Knight et al. (1981) with ppp5'A2'p5'2'p5'A2'p5'A3'[^{32}P]5'Cp of specific activity 3000 Ci/mmol, unfractionated S30 mouse spleen extract being used as a source of $(2'-5')(A)_n$-dependent endoribonuclease (RNase L, EC 3.1.27).

Protein Synthesis Assay. L1210 cell cultures (2.5×10^5 cells) were washed in methionine-free RPMI 1640 medium and labeled for 30 min at 37 °C with 10 μCi of [^{35}S]-methionine (sp act. 1109 Ci/mmol) in 0.25 mL of methionine-free medium supplemented by 5% (v/v) fetal calf serum at the times indicated in the individual experiments. After labeling, the medium was removed by centrifugation, and cells were washed twice with isotonic phosphate-buffered saline (pH 7.0) at 0 °C. The proteins were precipitated with 1 mL of 5% (w/v) trichloroacetic acid (TCA) for 1 h at 4 °C. The pellet was washed 3 times with 5% (w/v) TCA and solubilized at 100 °C for 3 min with 100 μL of 100 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.9) containing 10%

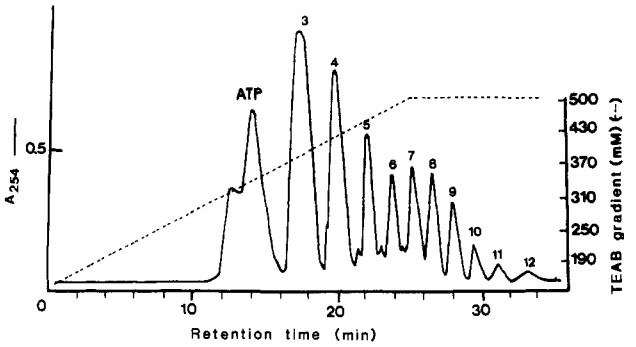

FIGURE 1: Preparative high-performance liquid chromatography of $(2'-5')(A)_n$ oligonucleotides. Cell-free extracts prepared from IFN-treated HeLa cells were incubated for 2 h at 37 °C with 5 mM ATP and 20 μg/mL poly(rI)·poly(rC). The proteins were precipitated, and the supernatant was loaded onto a TSK-DEAE column (LKB). $(2'-5')(A)_n$ oligomers were eluted with a linear gradient of 125–500 mM TEAB. The position of elution of ATP and $(2'-5')(A)_n$ oligomers of increasing lengths is indicated on the chromatogram.

(v/v) sodium dodecyl sulfate. Radioactivity (50 μL of the sample) was measured in 2 mL of HP/b liquid scintillation solvent (Beckman). In some experiments, ^{35}S-labeled proteins were analyzed by 10% (w/v) polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described by Laemmli (1970). Labeled proteins were located by fluorography after exposure to 10% (v/v) 2,5-diphenyloxazole in dimethyl sulfoxide (Laskey & Hills, 1975).

Assay for Ribosomal RNA Cleavage in Intact Cells. A total of 5×10^6 L1210 cells was incubated with $(2'-5')(A)_4$–poly(L-lysine) as indicated in the legends of the figures. The cells were then washed twice with phosphate-buffered saline (PBS) and collected by centrifugation, swollen for 10 min at 0 °C in hypotonic buffer (1 mM Tris-HCl, pH 7.4, 1 mM NaCl, and 0.5 mM $MgCl_2$), and disrupted by repeated pipetting with a P200 Gilson Pipetman. One-tenth volume of a buffer containing 100 mM Tris-HCl, pH 7.4, 1 M NaCl, and 10 mM ethylenediaminetetraacetic acid (EDTA) was added, and the nuclei were pelleted at 1100*g* for 10 min (Hugues & Robins 1983). The RNA was precipitated with ethanol overnight at −20 °C. Denatured cytoplasmic RNA (10 μg/sample) isolated from either controls or $(2'-5')(A)_4$–poly(L-lysine) conjugate treated L1210 cells was analyzed by electrophoresis in 1.2% (w/w) agarose slab gels in 2 mM EDTA and 89 mM Tris–boric acid buffer (pH 8.0). After electrophoresis at 50 mA for 2 h, the gels were stained with ethidium bromide, and the RNA bands were examined in a UV light box.

RESULTS

Conjugation of $(2'-5')(A)_n$ and Poly(L-lysine). It is well-known that the 5′-terminus phosphorylated moiety markedly influences $(2'-5')(A)_n$ oligonucleotide binding and activation of RNase L (Martin et al., 1979; Baglioni et al., 1981). On the other hand, 2′-phosphodiesterase digests $(2'-5')(A)_n$ from its 2′-terminus where free 2′- and 3′-hydroxy groups promote its degradation (Eppstein et al., 1982; Imai et al., 1982; Bayard et al., 1984). $(2'-5')(A)_4$ was then oxidized at the 2′-terminal ribose residue and coupled to ε-amino groups of poly(L-lysine) by an amino reductive reaction (Khym, 1963; Imai et al., 1982).

$(2'-5')(A)_n$ oligomers were synthesized enzymatically in IFN-treated HeLa cell extracts and fractionated in a single step by HPLC ion-exchange chromatography on a TSK-DEAE preparative column as described under Experimental Procedures. This procedure makes it possible to prepare milligram amounts of pure oligomers in less than 1 h (Figure 1). After periodate oxidation, the $(2'-5')(A)_4$ tetramer was coupled to M_r 14000 poly(L-lysine). The introduction of two aldehyde functions at the 2′ end of the molecule by periodate oxidation of the α-glycol group of the ribose residue was carried out quantitatively at 4 °C for 30 min, and the kinetics of the reaction was monitored by HPLC on a C_{18} μBondapak column [the retention times of $(2'-5')(A)_4$ and oxidized $(2'-5')(A)_4$ were 10 and 9 min, respectively].

Table I: Antiviral Activity of Poly(L-lysine)-Conjugated $(2'-5')(A)_4$

expt	$(2'-5')(A)_n$ conjugate	poly(L-lysine) concn (nM)	% of virus yield in control cells[a]
1[b]	poly(L-lysine)	500	100
	poly(L-lysine) + $(2'-5')(A)_3$2′P(trihydroxy acyclic A)	500	100
	poly(L-lysine)–$(2'-5')(A)_4$ core	500	35
	poly(L-lysine)–$(2'-5')(A)_4$	500	3
2[c]	poly(L-lysine)–$(2'-5')(A)_4$	500	1
	poly(L-lysine)–$(2'-5')(A)_4$	180	0.6
	poly(L-lysine)–$(2'-5')(A)_4$	100	5.0
	poly(L-lysine)–$(2'-5')(A)_4$	36	100

[a] Cells have been infected with VSV (m.o.i. 1) 3 h after incubation of L1210 cells with the conjugate. [b] A total of 2 mol of $(2'-5')(A)_n$ coupled to 1 mol of poly(L-lysine). [c] A total of 5 mol of $(2'-5')(A)_n$ coupled to 1 mol of poly(L-lysine).

The two aldehyde groups were coupled to ε-amino groups of lysine residues of poly(L-lysine) by Schiff base formation and reduced with sodium cyanoborohydride at pH 8.0 (Khym, 1963). This procedure, which converts the ribose ring into a morpholine structure (Brown & Reed, 1985), has been used successfully in immunochemistry (Erlanger, 1980), in $(2'-5')(A)_n$ derivative constructions (Imai et al., 1982), and in the preparation of neoglycoproteins (Lee et al., 1980; Menche & Wold, 1982). The $(2'-5')(A)_n$–poly(L-lysine) conjugates were then isolated by gel filtration chromatography on a Sephadex G-50 column.

Antiviral Activity of $(2'-5')(A)_n$–Poly(L-lysine) Conjugates. $(2'-5')(A)_n$ tetramers were conjugated to poly(L-lysine) with different ratios (two or five residues of $(2'-5')(A)_n$ per polypeptide chain) and incubated with murine leukemic L1210 cells 3 h prior to infection with vesicular stomatitis virus (VSV) at a multiplicity of infection (m.o.i.) of 1. Virus yields were determined 24 h later by conventional assays and expressed as a percentage of the yield obtained in untreated cells. As summarized in Table I, both preparations of conjugates resulted in dose-dependent reduction of the production of VSV by over 99% at a conjugate concentration of 180 nM, i.e., a concentration of $(2'-5')(A)_n$ inferior to 1 μM. Additional experiments will be required to determine the intracellular concentrations of $(2'-5')(A)_n$–poly(L-lysine) conjugate that are achieved by this procedure. Poly(L-lysine) itself did not affect virus production even at the highest concentration (500 nM) used in our experiments. This agrees with data obtained by Ryser and Hancock (1965), reporting cytotoxicity in vitro with amounts greater than 1 μM only. Neither $(2'-5')(A)_n$ alone nor a mixture of poly(L-lysine) and $(2'-5')(A)_n$ (in the same molar ratio as in the conjugates) had any effect on virus yield as would be expected given the absence of penetration of the highly charged $(2'-5')(A)_n$ oligomers in intact cells. $(2'-5')(A)_4$ analogues prepared by periodate oxidation followed by borohydride reduction of $(2'-5')(A)_n$, as described by Bayard et al. (1984), were used in this latter test. These 3′-modified $(2'-5')(A)_n$ analogues displayed the increased metabolic stability and antiviral activity (Bayard et al., 1984, 1985a,b) expected from $(2'-5')(A)_4$–poly(L-lysine) conjugates

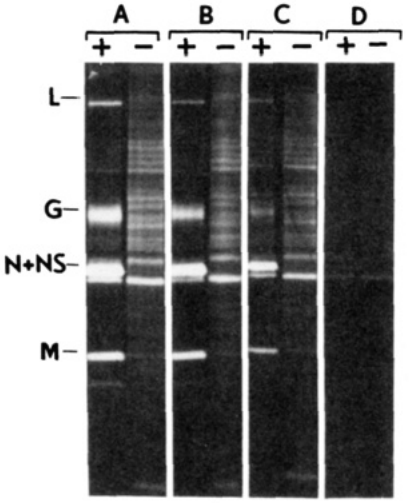

FIGURE 2: PAGE–SDS analysis of the inhibition of protein synthesis by (2′–5′)$(A)_4$–poly(L-lysine) in uninfected (–) or VSV infected (+) L1210 cells. Cells were infected with 5 m.o.i. of VSV and treated with 1×10^{-6} M [in (2′–5′)$(A)_4$] of the conjugate for 14 h. Incorporation of [^{35}S]methionine into acid-insoluble material was analyzed in PAGE–SDS as described under Experimental Procedures. The total acid-insoluble radioactivity incorporated in 2.5×10^5 cells was analyzed for each sample. An autoradiograph of the dried gel is shown: (A) uninfected (–) or VSV-infected (+) cells treated with 2.1×10^{-7} M poly(L-lysine) and 1×10^{-6} M (2′–5′)$(A)_4$; (B) untreated cells; (C) cells treated with dephosphorylated or core (2′–5′)$(A)_4$–poly(L-lysine) [1×10^{-6} M in (2′–5′)$(A)_4$] conjugate; (D) cells treated with (2′–5′)$(A)_4$–poly(L-lysine) [1×10^{-6} M in (2′–5′)$(A)_4$]. M, NS, N, G, and L at the left of the gel indicate the position of VSV proteins; their molecular weights were 34 000, 47 200, 52 480, 67 300, and 151 000, respectively.

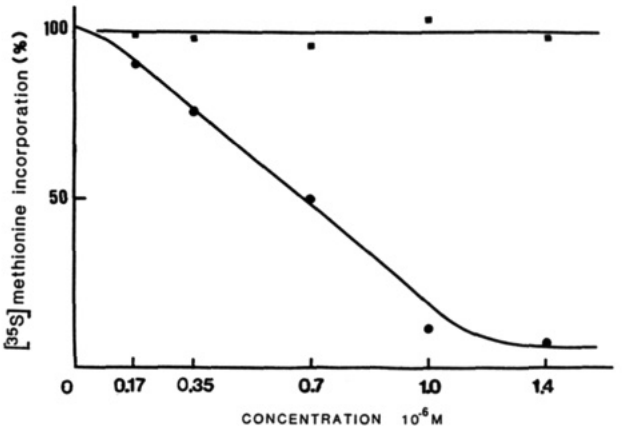

FIGURE 3: Effect of (2′–5′)$(A)_4$–poly(L-lysine) conjugates on protein synthesis in intact L1210 cells. A total of 2.5×10^5 L1210 cells in suspension culture was treated with the (2′–5′)$(A)_4$ tetramer conjugate at doses of 1.4×10^{-6}, 1×10^{-6}, 7×10^{-7}, 3.5×10^{-7}, and 1.75×10^{-7} M in (2′–5′)$(A)_4$ for 3 h (●) or with a mixture of (2′–5′)-$(A)_4$-stabilized tetramer and poly(L-lysine) (■) at the same concentrations. The mean ratio of coupling of (2′–5′)$(A)_4$ to poly(L-lysine) was 5:1 in this experiment. Protein synthesis in response to exogenously added conjugate in cell culture was assessed by the inocrporation of [^{35}S]methionine for 30 min by the procedure described under Experimental Procedures.

or from the products of their intracellular metabolism. Surprisingly, dephosphorylated or "core" (2′–5′)$(A)_4$–poly(L-lysine) conjugates had a significant antiviral activity although much less than phosphorylated (2′–5′)$(A)_n$–poly(L-lysine) conjugates at the same final concentration.

Protein Synthesis in (2′–5′)$(A)_n$–Poly(L-lysine)-Treated, VSV-Infected Cells. It was of interest to determine whether there was a correlation between the inhibition of virus yield and activation of RNase L, since the activation of this enzyme at subnanomolar concentrations of (2′–5′)$(A)_n$ cleaves mRNAs and rRNAs with consequent inhibition of protein synthesis. If poly(L-lysine) does in fact serve as a transmembrane carrier of (2′–5′)$(A)_n$, these conjugates should lead to an inhibition of protein synthesis, as demonstrated previously with liposome-encapsulated (2′–5′)$(A)_n$ and (2′–5′)$(A)_n$ analogues (Bayard et al., 1985a,b). Cells were incubated with 10^{-6} M (2′–5′)$(A)_4$–poly(L-lysine) [in (2′–5′)$(A)_4$] for 90 min and infected with VSV at a m.o.i of 5 for 14 h. Cells were then pulse-labeled with L-[^{35}S]methionine for 30 min, and acid-insoluble polypeptides were analyzed by electrophoresis in polyacrylamide gels (PAGE–SDS) as shown in Figure 2. As can be seen from the comparison of lanes A and B, a mixture of unconjugated stabilized (2′–5′)$(A)_4$ (1×10^{-6} M) and poly(L-lysine) (2.1×10^{-7} M) did not affect cellular protein synthesis or prevent the virus-induced shut off of host protein synthesis. The five viral proteins L, G, NS, N, and M (respective M_r 151 000, 67 300, 52 500, 47 200, and 34 000) were well characterized. Slightly reduced [^{35}S]methionine incorporation was obtained with 1×10^{-6} M "core" (2′–5′)$(A)_4$–poly(L-lysine) (lane C; this is a possible explanation for its weak antiviral activity). Dephosphorylated core (2′–5′)$(A)_4$–poly-(L-lysine) did bind RNase L although to a much lesser extent (IC_{50} 1×10^{-6} M) than phosphorylated (2′–5′)$(A)_4$ (IC_{50} 2×10^{-8} M). In contrast, and in agreement with binding data (vide infra), substantial (>90% inhibition of protein synthesis (lane D) was obtained with (2′–5′)$(A)_4$–poly(L-lysine) (1.0×10^{-6} M) in VSV-infected L1210 cells. (2′–5′)$(A)_4$–poly-(L-lysine), which inhibits VSV replication, reduced protein synthesis in infected and uninfected cells to the same extent.

Inhibiton of Protein Synthesis in Intact L1210 Cells. (2′–5′)$(A)_4$ conjugated to poly(L-lysine) in a molar ratio of 5:1 inhibited cellular protein synthesis in L1210 cells in a dose-dependent manner as measured by the incorporation of [^{35}S]methionine in acid-precipitable material (Figure 3). After incubation for 3 h, (2′–5′)$(A)_4$–poly(L-lysine) gave rise to inhibition of protein synthesis with an ED_{50} of 7×10^{-7} M [external medium concentration estimated in (2′–5′)$(A)_4$]. Substantial inhibition of protein synthesis (>90%) had already been obtained at 1×10^{-6} M. Core (2′–5′)$(A)_4$ did not bind and activate RNase L well (Knight et al., 1981) and thus caused very slight protein synthesis inhibition when associated with the poly(L-lysine) carrier. Unbound (2′–5′)$(A)_4$ added to poly(L-lysine) as a control did not inhibit protein synthesis since (2′–5′)$(A)_n$ cannot penetrate intact cells, as reported previously by Hovanessian et al., (1979).

The kinetics of protein synthesis inhibition in cells incubated with (2′–5′)$(A)_4$–poly(L-lysine) (ratio 5:1) at different doses are shown in Figure 4. As expected, the inhibitory effect of (2′–5′)$(A)_4$–Poly(L-lysine) conjugates on protein synthesis developed more rapidly at higher doses. For instance, ED_{50} at 1×10^{-6} M was reached after 1 h while ED_{50} at 7×10^{-7} M was obtained 3 h after the start of incubation. The maximum inhibitory effect was obtained 3–6 h after exposure of cells to the conjugates and lasted until 15–18 h thereafter. However, cellular protein synthesis returned to nearly normal levels 24 h after the start of incubation.

Binding of (2′–5′)$(A)_4$–Poly(L-lysine) Conjugate to RNase L. We used the radiobinding assay described by Knight et al. (1981) to make a direct approach to the ability of (2′–5′)$(A)_n$–poly(L-lysine) conjugates to bind RNase L. This procedure is based on the ability of conjugated (2′–5′)$(A)_n$ oligomers to compete with a ^{32}P-labeled (2′–5′)$(A)_n$ probe for its specific binding to the (2′–5′)$(A)_n$-dependent endonuclease in an unfractionated cell-free extract, prepared in this case from bovine spleen. Displacement curves are illustrated in

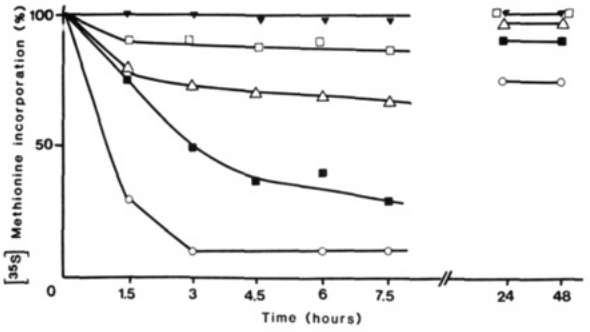

FIGURE 4: Kinetics of inhibition of protein synthesis by (2′–5′)-$(A)_4$–poly(L-lysine) in L1210 cell culture. Cells were treated with 1.75×10^{-7} (□), 3.5×10^{-7} (△), 7×10^{-7} (■), and 1×10^{-6} M (○) (2′–5′)$(A)_4$–poly(L-lysine) [values are expressed in (2′–5′)$(A)_4$ concentration] as in the experiment described in Figure 3. Protein synthesis was monitored after 1.5–24 h of incubation by labeling with [^{35}S]methionine for 30 min and determining the amount of acid-precipitable radioactivity as described under Experimental Procedures. A mixture of poly(L-lysine) (2.8×10^{-7} M) and (2′–5′)$(A)_4$ (1.4×10^{-6} M) stablized at its 2′ end [trihydroxy acyclo (2′–5′)$(A)_4$] was used as a control (▼), and the results are expressed as a percentage of untreated cells.

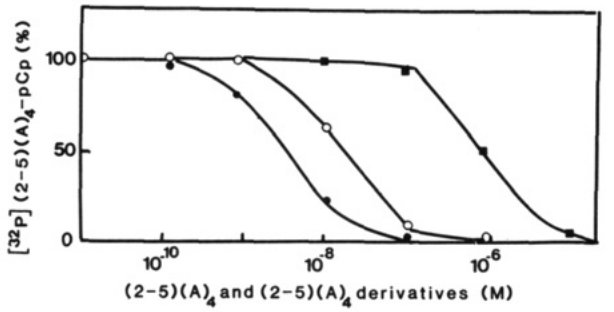

FIGURE 5: Radiobinding assay. Ability of unconjugated (2′–5′)$(A)_4$ or (2′–5′)$(A)_4$ conjugates to compete with [^{32}P](2′–5′)$(A)_n$pCp probe for its binding to the RNase L of mouse spleen S30 extracts according to the procedure of Knight et al. (1980): Free (2′–5′)$(A)_4$ (●); (2′–5′)$(A)_4$–poly(L-lysine) (○); dephosphorylated or core (2′–5′)-$(A)_4$–poly(L-lysine) (■).

Figure 5. As previously defined by Imai et al. (1982), the results can be expressed in terms of IC_{50}, i.e., the concentrations required to inhibit 50% of the binding of the [^{32}P](2′–5′)$(A)_n$pCp probe. Under our experimental conditions, the results demonstrate that (2′–5)$(A)_4$ conjugated to poly(L-lysine) binds RNase L with less affinity (IC_{50} of 2×10^{-8} M) than unbound (2′–5′)$(A)_4$ (IC_{50} of 5×10^{-9} M). This latter result is similar to values reported by others (Knight et al., 1981). Dephosphorylation of the 5′-terminal triphosphate moiety resulted in a dramatic decrease in the ability to bind RNase L [IC_{50} of core (2′–5′)$(A)_4$–poly(L-lysine) 10^{-6} M].

Activation of RNase L by (2′–5′)$(A)_n$–Poly(L-lysine) Conjugate. As shown above for the in vitro radiobinding assay, (2′–5′)$(A)_4$ conjugates bound RNase L with less affinity than unconjugated (2′–5′)$(A)_4$. It was therefore considered important to find out whether (2′–5′)$(A)_4$–poly(L-lysine) conjugates activate RNase L in intact cells. This enzyme is known to degrade ribosomal RNAs (rRNAs) into highly characteristic cleavage products (Wreshner et al., 1981b) when activated by (2′–5′)$(A)_n$ or (2′–5′)$(A)_n$ analogues introduced in intact cells by calcium phosphate coprecipitation. L1210 cells were thus incubated with (2′–5′)$(A)_4$–poly(L-lysine), and total cytoplasmic RNA was isolated and analyzed by electrophoresis on agarose gels under denaturing conditions. The pattern of cleavage products obtained after incubation for 3 h with increasing doses of (2′–5′)$(A)_n$ conjugates is shown in Figure 6. The location of the undegraded 18S and 28S rRNA bands

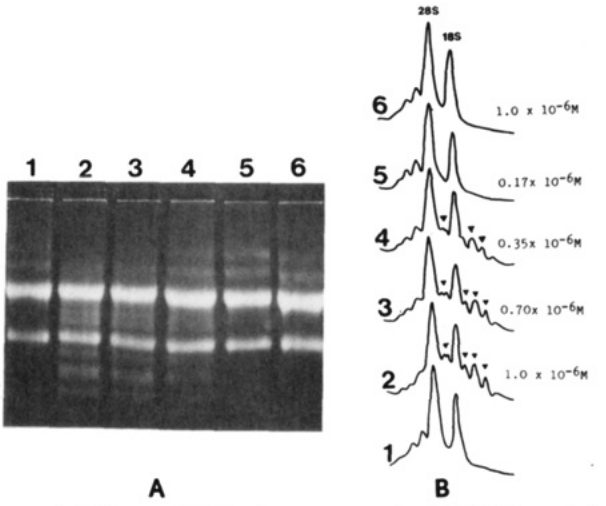

FIGURE 6: Ribosomal RNA cleavage assay by (2′–5′)$(A)_4$–poly(L-lysine) conjugate in intact L1210 cells. (A) Cells were treated for 2 h with different doses of the conjugate; RNA was extracted, denatured, and analyzed in 1.2% agarose gel electrophoresis as described under Experimental Procedures. The agarose gel was stained with ethidium bromide for 1 h and viewed under ultraviolet light. Migrations of the 28S and 18S rRNAs as well as the location of the major (2′–5′)$(A)_n$-specific rRNA cleavage products are shown by the arrows. (B) Densitometric tracing of tracks from (A): (track 1) no additions, control; (tracks 2–5) L1210 cells treated for 2 h with 1×10^{-6}, 0.7×10^{-6}, 0.35×10^{-6}, and 0.17×10^{-6} M (2′–5′)$(A)_4$–poly(L-lysine) conjugates [concentrations in (2′–5′)$(A)_4$]; (track 6) cells treated with dephosphorylated core (2′–5′)$(A)_4$–poly(L-lysine) conjugate [1×10^6 M in (2′–5′)$(A)_n$].

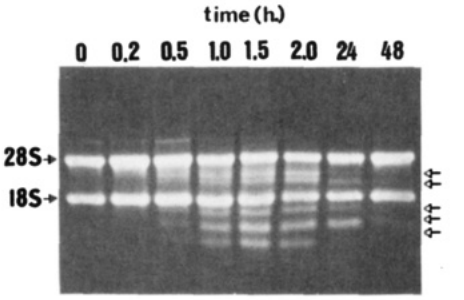

FIGURE 7: Kinetics of rRNA cleavage in L1210 cells treated with the (2′–5′)$(A)_4$–poly(L-lysine) conjugate. Cells were treated at the times specified with the conjugate [1×10^{-6} M in (2′–5′)$(A)_n$] and processed as described in the legend of Figure 6. A photograph of the ethidium bromide stained gel taken under UV light (302 nM) is provided. The position of 28S and 18S rRNAs and the five major products of (2′–5′)$(A)_4$–poly(L-lysine)-specific rRNA cleavage are indicated by arrows at the left and right of the gel, respectively.

is given in track 1, which is for untreated cells used as a control. (2′–5′)$(A)_4$–poly(L-lysine) gave rise to significant, dose-dependent rRNA cleavages with concentrations as low as 0.35×10^{-6} M (tracks 2–4), agreeing with protein synthesis inhibition data. No further significant degradation products were detected at the lowest concentration of 0.17×10^{-6} M (track 5). Dephosphorylated core (2′–5′)$(A)_4$–poly(L-lysine) failed to induce a degradative pattern (track 6). There is a striking similarity between the pattern of degradation of rRNAs observed here and earlier data obtained with unconjugated (2′–5′)$(A)_n$ introduced in intact cells by the calcium phosphate coprecipitation technique (Wreshner et al., 1981b).

The activation of RNase L under our experimental conditions was transient, like protein synthesis inhibition. L1210 cells were incubated with (2′–5′)$(A)_4$–poly(L-lysine) in a 5:1 ratio at 1×10^{-6} M in (2′–5′)$(A)_n$. Ribosomal RNA was extracted at various times after incubation and analyzed by agarose gel electrophoresis. As shown in Figure 7, the rRNA

cleavages began to be detectable after 30 min and reached their most intense levels 90 min later. This phenomenon persisted for 24 h and disappeared 48 h after incubation.

Discussion

(2′–5′)$(A)_4$ was coupled through its oxidized 3′ end to ε-amino groups of poly(L-lysine) with a mean M_r of 14000. Although most of the experiments described here concern (2′–5′)$(A)_4$ conjugated to poly(L-lysine) in a molar ratio of 5:1, different levels of lysine substitution were obtained essentially by varying the relative concentrations of (2′–5′)$(A)_4$ and poly(L-lysine) in the coupling reaction. The extent to which this and other parameters like the molecular weight of the polypeptide carrier or the length of the (2′–5′)$(A)_n$ chain influence the properties of the conjugates has not yet been investigated thoroughly. (2′–5′)$(A)_4$ was chosen initially since its coupling leaves unchanged at least the three 2′–5′-linked adenylic residues required for optimal recognition and activation of RNase L [reviewed by Johnston & Torrence (1984)].

As can be seen from the biological activity of (2′–5′)-$(A)_4$–poly(L-lysine) conjugates, poly(L-lysine) forms an effective vector for the intracellular transport of functional (2′–5′)$(A)_n$. The activity of the conjugates is most probably linked with the activation of the (2′–5′)$(A)_n$-dependent RNase L either by the conjugate itself or by intracellular release of (2′–5′)$(A)_n$. (2′–5′)$(A)_4$–Poly(L-lysine) conjugates do compete with a radioactive probe for binding to RNase L in the radiobinding assay described by Knight et al. (1981). Their lesser affinity compared to unconjugated (2′–5′)$(A)_4$, probably results from steric hindrance with the polypeptide chain. Whatever the explanation, this is not necessarily a problem since, for instance, methotrexate (MTX)–poly(L-lysine) conjugates have been shown to be active in intact cells while unable to inhibit dihydrofolate reductase in a cell-free assay (Ryser & Shen, 1978).

Additional arguments for the intracellular activation by (2′–5′)$(A)_4$–poly(L-lysine) conjugates of RNase L stem from the dose-dependent, time-dependent, and reversible inhibition of protein synthesis described in Figures 2–4. The nondiscriminated inhibition of both viral and cellular protein synthesis in VSV-infected cells is not unexpected since the specificity of RNase L probably originates from its localized activation in IFN-treated virus-infected cells (Nilsen & Baglioni, 1979) rather than from its recognition site. It is reminded that partially purified RNAse L preparations available cleave their RNA substrates on the 3′ side of UpX sequences (Floyd-Smith et al., 1981; Wreschner et al., 1981a). The reversibility of protein synthesis inhibition and of rRNA cleavage (vide infra) observed here is probably connected with the degradation of (2′–5′)$(A)_n$ itself by phosphatases. Phosphodiesterases, such as the 2′PDI described by Schmidt et al. (1979), are probably not involved here since conjugation to poly(L-lysine) introduces an *N*-morpholine ring at the 3′ end of (2′–5′)$(A)_n$ that like most modifications at this end of the molecule [reviewed by Johnston & Torrence (1984)], protects them from the processive action of phosphodiesterases. Measurements of the intracellular metabolism of these and of phosphatase-resistant 5′-modified (Bayard et al., 1985) (2′–5′)$(A)_n$–poly(L-lysine) conjugates will obviously be worthwhile in further studies. Likewise, (2′–5′)$(A)_n$ or (2′–5′)$(A)_n$ analogues introduced in intact cells by calcium phosphate coprecipitation (Hovanessian & Wood, 1980), microinjection with micropipets (C. Bisbal et al., unpublished results), or liposome encapsulation (Bayard et al., 1985) lead to reversible inhibition of protein synthesis, although kinetics and dose dependencies are different. Direct comparison of effective doses would be irrelevant since actual intracellular (2′–5′)$(A)_n$ concentrations were not measured in any of these studies.

Finally, (2′–5′)$(A)_n$–poly(L-lysine) conjugates [but not free (2′–5′)$(A)_4$, mixtures of unconjugated (2′–5′)$(A)_4$ and poly(L-lysine), or "core" (2′–5′)$(A)_n$–poly(L-lysine) conjugates] do promote a dose-dependent set of cleavages of rRNAs, which has been shown to be characteristic of RNase L action on ribosomes (Wreshner et al., 1981b) and which forms the most direct argument at present available for RNase L activation in intact cells. This effect on rRNA cleavages is reversible, although at a lower rate than on protein synthesis. This is not unexpected since rRNA has a slow turnover rate.

While (2′–5′)$(A)_4$–poly(L-lysine) conjugates obviously promote activation of RNase L, and if this is probably the basis for their antiviral activity, questions remain regarding the route followed to transfer (2′–5′)$(A)_n$ from the culture medium to the interior of the cell. Since VSV replicates in cell cytoplasm and since ribosome cleavages occur, (2′–5′)$(A)_n$ has to reach the cytoplasmic compartment. Conjugation is necessary as free (2′–5′)$(A)_4$ or mixtures of (2′–5′)$(A)_4$ and poly(L-lysine) do not promote any of the biological activities reported above. The lag time observed before the onset of protein synthesis inhibition or rRNA cleavages is probably linked to the time necessary for the conjugate to cross the plasma membrane and for the intracytoplasmic delivery of (2′–5′)$(A)_n$, whatever the mechanism. Lysosomotropic drugs like mepacrine (used at 3 μM) and chloroquine (used here at 100 μM) do not inhibit (2′–5′)$(A)_4$–poly(L-lysine)-mediated rRNA cleavage (data not shown). This also applies to MTX–poly(L-lysine) conjugates (H. P. Ryser, personal communication). This suggests but by no way proves that these conjugates either enter cells through a nonendocytic pathway or escape from the acidic compartment before reaching lysosomes at the endosome level for example. Since (2′–5′)$(A)_4$ does bind RNase L effectively in its conjugated form as described above, it is not known whether degradation of the polypeptide moiety of the conjugate actually takes place as has been proposed for MTX–poly(L-Lysine) conjugates (Chen & Ryser, 1978).

In short, and although several parameters might still be considered to optimalize delivery, poly(L-lysine) is an efficient vector for (2′–5′)$(A)_4$. It will also be useful to deliver and to study the metabolism and the role of agonists and antagonists of the RNase L pathway introduced in this way in intact cells and tissues, and possibly also for the delivery of other oligonucleotides of biological interest. No cytotoxicity of poly(L-lysine) itself was observed at the doses used in these in vitro experiments. In vivo, however, poly(L-lysine) might generate toxicity (H. P. Ryser, personal communication) unless chelated by polyanionic substances like heparin (Shen & Ryser, 1981) or carboxymethylcellulose, as successfully used for the poly(rI)·poly(rC) IFN inducers now undergoing clinical trials [reviewed in Levy (1980)]. This technology could thus be used to elucidate the role of the (2′–5′)$(A)_n$ pathway in vivo, as for instance in the antiviral protection provided by exogenous or endogenous IFNs, and to examine its possible utilization in antiviral and antineoplasic chemotherapy. Alternatively, these or similar techniques might conceivably be used to make a covalent link between (2′–5′)$(A)_n$ and natural or synthetic polypeptides, allowing targeting of the conjugated drug. Coupling to asialoglycoproteins as reviewed by Ashwell and Harford (1982) might, for instance, allow one to introduce (2′–5′)$(A)_n$ preferentially into hepatocytes.

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REFERENCES

Ashwell, G., & Harford, T. (1982) *Annu. Rev. Biochem. 51*, 531–554.

Baglioni, C., D'Allessandro, S. B., Nilsen, T., Den Hartog, J. A., Crea, R., & Van Boom, J. M. (1981) *J. Biol. Chem. 256*, 3253–3257.

Bayard, B., Bisbal, C., Silhol, M., Cnockaert, J., Huez, G., & Lebleu, B. (1984) *Eur. J. Biochem. 142*, 291–298.

Bayard, B., Leserman, L. D., Bisbal, C., Huez, G., Silhol, M., & Lebleu, B. (1985a) *Nucleosides Nucleotides 4*, 157–160.

Bayard, B., Leserman, L. D., Bisbal, C., & Lebleu, B. (1985b) *Eur. J. Biochem. 151*, 319–325.

Brown, D. M., & Reed, A. P. (1965) *J. Chem. Soc.*, 5072–5074.

Brown, G. E., Lebleu, B., Kawakita, M., Shaila, S., Sen. G. C., & Lengyel, P. (1976) *Biochem. Biophys. Res. Commun. 69*, 114–122.

Brown, R. E., Cayley, P. J., & Kerr, I. (1981) *Methods Enzymol. 79*, 208–216.

Eppstein, D., Marsch, Y. V., Schryver, B. B., Larsen, M. A., Barnett, J. W., Verheyden, J. P., & Prisbe, E. J. (1982) *J. Biol. Chem. 257*, 13390–13397.

Eppstein, D., Marsch, Y. V., & Schryver, B. B. (1983) *Virology 131*, 341–354.

Erlanger, B. F. (1980) *Methods Enzymol. 70*, 85–104.

Etienne-Smekens, M., Vandenbussche, P., Content, J., & Dumont, J. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4609–4613.

Floyd-Smith, G., Slattery, E., & Lengyel, P. (1981) *Science (Washington, D.C.) 212*, 1030–1032.

Higashi, Y., & Sokawa, Y. (1982) *J. Biochem. (Tokyo) 91*, 2021–2028.

Hovanessian, A. G., & Wood, J. N. (1980) *Virology 101*, 81–90.

Hovanessian, A. G., Wood, J., Meurs, E., & Montagnier, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3261–3265.

Hughes, B., & Robins, R. (1983) *Biochemistry 22*, 2127–2135.

Imai, J., & Torrence, P. F. (1984) *Biochemistry 23*, 766–774.

Imai, J., Johnston, M. I., & Torrence, P. F. (1982) *J. Biol. Chem. 257*, 12739–12741.

Johnston, I. M., & Torrence, P. F. (1984) in *Interferon* (Friedman, R. M., Ed.) Vol. 3, pp 189–298, Elsevier, Ameterdam.

Keller, W. (1984) *Cell (Cambridge, Mass.) 38*, 299–302.

Kerr, I. M., & Brown, R. E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 256–280.

Khym, J. C. (1963 *Biochemistry 2*, 344–350.

Kimchi, A., Shure, M., & Revel M. (1981) *Eur. J. Biochem. 114*, 5–10.

Knight, M., Wrechner, D. H., Silverman, R. M., & Kerr, I. M. (1981) *Methods Enzymol. 79*, 216–222.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Laskey, R., & Hills, A. (1975) *Eur. J. Biochem. 56*, 335–341.

Lebleu, B., & Content, J. (1982) in *Interferon* (Gresser I., Ed.) Vol. 4, pp 47–94, Academic, New York.

Lee, R. T., & Lee, Y. C. (1980) *Biochemistry 19*, 156–163.

Levy, H. B. (1980) in *Interferon and Interferon Inducers: Clinical Applications* (Stringfellow, D. A., Ed.) pp 167–186, Dekker, New York.

Lowry, O. H., Rosebrough, N. S., Farr, A. L., & Randall, R. I. (1951) *J. Biol. Chem. 193*, 265–275.

Martin, E. M., Birdsall, N. S., Brown, R. E., & Kerr, I. M. (1979) *Eur. J. Biochem. 95*, 295–307.

Mencke, A., & Wold, F. (1982) *J. Biol. Chem. 257*, 14799–14805.

Minks, M. A., Benvin, S., Maroney, P. A., & Baglioni, C. (1979) *J. Biol. Chem. 254*, 5058–5064.

Nilsen, T. W., & Baglioni, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2600–2604.

Nilsen, T. W., Wood, D. L., & Baglioni, C. (1982) *J. Biol. Chem. 257*, 1602–1605.

Reid, T. R., Hersh, C. L., Kerr, I. M., & Stark, G. R. (1984) *Anal. Biochem. 136*, 136–141.

Roberts, W. K., Hovanessian, A., Brown, R. E., Clemens, M. J., & Kerr, I. M. (1976) *Nature (London) 264*, 477–480.

Ryser, H. P., & Hancock, R. (1965) *Science (Washington, D.C.) 150*, 501–503.

Ryser, H., & Shen, W. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3867–3870.

Shen, W. C., & Ryser, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1872–1876.

Shen, W. C., & Ryser, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7589–7593.

Silverman, R. H. (1984) in *Interferon* (Friedman, R. M., Ed.) Vol. 3, pp 177–188, Elsevier, Amsterdam.

Stark, G. R., Dower, W. J., Schimke, R. T., Brown, R. E., & Kerr, I. M. (1979) *Nature (London) 278*, 471–473.

St Laurent, G., Yoshie, O., Floyd-Smith, G., Samanta, H., Sehgal, P. B., & Lengyel, P. (1983) *Cell (Cambridge, Mass.) 33*, 95–102.

Watling, D., Serafinowska, H. T., Reese, C. B., & Kerr, I. M. (1985) *EMBO J. 4*, 431–436.

Williams, B. R. G., & Kerr, I. M. (1978) *Nature (London) 176*, 88–90.

Wreschner, D. M., Mc Cauley, J. W., Skehel, J. J., & Kerr, I. M. (1981a) *Nature (London) 289*, 414–417.

Wreschner, D. H., James, T. C., Silverman, R. H., & Kerr, I. M. (1981b) *Nucleic Acids Res. 9*, 1571–1578.

CORRECTION

Human Serum Amyloid A (SAA): Biosynthesis and Post-synthetic Processing of PreSAA and Structural Variants Defined by Complementary DNA, by J. D. Sipe, H. R. Colten,* G. Goldberger, M. D. Edge, B. F. Tack, A. S. Cohen, and A. S. Whitehead, Volume 24, Number 12, June 4, 1985, pages 2931–2936.

Page 2933. In Figure 2, the derived amino acid at position 63 should be E. Also, the nucleotide sequence coding for position 101 should be CCT and the derived amino acid at position 101 should be P.