# ACTIVATION OF HUMAN AND MOUSE 2-5A SYNTHETASES AND MOUSE PROTEIN $P_1$ KINASE BY NUCLEIC ACIDS

## Structure–activity relationships and correlations with inhibition of protein synthesis and interferon induction

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### 1. Introduction

The natural occurrence of fully double-stranded RNA (dsRNA) of high molecular weight in viruses, virus-like particles in fungi, as intermediates in the replication of RNA and DNA viruses has suggested some possible biological role for such molecules [1]. Interest in dsRNA as a possible regulatory signal has been generated by the observations that dsRNA is a potent interferon inducer [2] and a powerful inhibitor of protein synthesis in extracts of rabbit reticulocytes and of interferon-treated cells [3]. Particularly fascinating is the fact that interferon-pretreatment of cells gives cell-free extracts which have enhanced sensitivity to inhibition of translation by dsRNA [4]. Two separate pathways may mediate the protein synthesis inhibitory effects of dsRNA [5–7]: the first pathway is referred to as the 2–5A pathway and involves the activation by dsRNA of an enzyme referred to 2–5A synthetase which synthesizes from ATP a mixture of 2′,5′-linked oligonucleotides called 2–5A [8]. This oligonucleotide then activates a latent endonuclease which degrades mRNA and rRNA resulting in an inhibition of protein synthesis [5–7]. A second distinct pathway is termed the protein kinase pathway. In this case, dsRNA activates a protein kinase which phosphorylates a 65–67 K dalton protein (protein $P_1$) and the small ($\alpha$) subunit of eIF-2. In this case, phosphorylation of eIF-2$\alpha$ is believed to decrease, either directly or indirectly, its ability to engage in initiation complex formation with a 40 S ribosomal subunit, GTP and Met-tRNA [3].

In this study, we report the relationships between the structure of a nucleic acid and its ability to activate the 2–5A synthetase and protein $P_1$ kinase. Since earlier studies have dealt with the dependence of interferon induction [2] and protein synthesis inhibition [9,10] on nucleic acid structure, it becomes possible to determine whether or not there may be any relationships among these various biological activities of dsRNA.

*Abbreviations:* dsRNA, double-stranded RNA; 2–5A refers to a series of 2′,5′-oligoadenylates of general formula $ppp(AP)_nA$ where n has been reported to be between 1 and approximately 10; $(I)_n$, polyinosinsic acid; $(C)_n$, polycytidylic acid; $(A)_n$, polyadenylic acid, $(U)_n$, polyuridylic acid; $(G)_n$, polyguanylic acid; $(m^7G)_n$, poly(7-methylguanylic acid); $(X)_n$, polyxanthylic acid; $(br^5C)_n$, poly(5-bromocytidylic acid); poly($c^7I$), poly(7-deazainosinic acids); $(s^2C)_n$, poly-(2-thiocytidylic acid); $(rT)_n$, polyribothymidylic acid; $(br^5U)_n$, poly(5-bromouridylic acid); $(fl^5U)_n$, poly(5-fluorouridylic acid); $(cl^5U)_n$, poly(5-chlorouridylic acid); poly($c^7A$), poly-(7-deazaadenylic acid); $(dUz)_n$, poly(2′-azido-2′-deoxyuridylic acid); $(dUfl)_n$, poly(2′-fluoro-2′-deoxyuridylic acid); $(Am)_n$, poly(2′-*O*-methyladenylic acid); $(Um)_n$, poly(2′-*O*-methyl-uridylic acid); $(Cm)_n$, poly(2′-*O*-methylcytidylic acid); $(dA)_n$, polydeoxyadenylic acid; $(dT)_n$, polydeoxythymidylic acid; $(dG)_n$, polydeoxyguanylic acid; $(dC)_n$, polydeoxycytidylic acid; $(dI)_n$, polydeoxyinosinic acid; $(dA\text{-}dT)_n$, an alternating copolymer of deoxyadenylic acid and deoxythymidylic acids; $(A-U)_n$, an alternating copolymer of riboadenylic acid and ribouridylic acid; EMCV, encephalomyocarditis virus; $M_p$, molar concentration based on phosphate; protein $P_1$, ribosome-associated protein of $M_r$ 65–67 000

Table 1
Activation of L Cell 2–5A Synthetase and L Cell Protein $P_1$ Kinase by Polynucleotides

| Polynucleotide | 2–5A Synthetase activity[a] | Protein $P_1$ kinase[b] | | Polynucleotide | 2–5A Synthetase activity[a] | Protein $P_1$ kinase[b] | |
|---|---|---|---|---|---|---|---|
| | | Buffer 1 | Buffer 2 | | | Buffer 1 | Buffer 2 |
| single-stranded[c] | <8 | – | – | base-modified | | | |
| | | | | $(X)_n \cdot (A)_n$ | <8 | – | – |
| triple-stranded | | | | $(X)_n \cdot (rT)_n$ | <8 | – | – |
| $(A)_n \cdot 2(U)_n$ | <8 | ++ | + | $(X)_n \cdot (br^5U)_n$ | <8 | – | – |
| $(A)_n \cdot (U)_n \cdot (I)_n$ | <8 | ++ | ND | $(c^7A)_n \cdot (U)_n$ | 280 | – | – |
| $(A)_n \cdot 2(I)_n$ | <8 | – | – | $(c^7A)_n \cdot (rT)_n$ | 80 | – | – |
| $(A)_n \cdot (X)_n \cdot (U)_n$ | <8 | ND | ++ | $(c^7A)_n \cdot (br^5U)_n$ | 80 | – | – |
| $(A)_n \cdot 2(X)_n$ | <8 | – | – | $(A)_n \cdot (rT)_n$ | <8 | ++ | ++ |
| | | | | $(A)_n \cdot (fl^5U)_n$ | <8 | – | + |
| alternating copolymer | | | | $(A)_n \cdot (cl^5U)_n$ | <8 | – | – |
| $(A\text{-}U)_n$ | 2000 | ND | ND | $(A)_n \cdot (br^5U)_n$ | <8 | – | + |
| | | | | $(A)_n \cdot (U)_n$ | <8 | ++ | ++ |
| natural dsRNAs | | | | | | | |
| *P. chrysogenum* | 8000 | ++ | ++ | sugar-modified | | | |
| killer yeast | 8000 | ++ | ++ | $(A)_n \cdot (dUz)_n$ | <8 | – | – |
| f2 bacteriophage | 8000 | ++ | ++ | $(A)_n \cdot (dUfl)_n$ | <8 | + | + |
| | | | | $(A)_n \cdot (Um)_n$ | <8 | ND | – |
| double-stranded | | | | $(Am)_n \cdot (U)_n$ | <8 | ND | – |
| double-sized dsRNA[d] | | | | $(Am)_n \cdot (Um)_n$ | <8 | ND | – |
| I(12.5) C(13.2) | 2800 | ++ | ++ | $(I)_n \cdot (Cm)_n$ | <8 | + | + |
| I(8) C(8.3) | 2800 | ++ | ++ | $(dA)_n \cdot (dT)_n$ | <8 | + | ND |
| I(2.5) C(3) | 2800 | ++ | ++ | $(dG)_n \cdot (dC)_n$ | <8 | ND | ND |
| I(12.5) C(3) | 2800 | ++ | ++ | $(dA\text{-}dT)_n$ | <8 | ND | – |
| I(2.5) C(13.2) | 2800 | ++ | ++ | $(dI)_n \cdot (dC)_n$ | <8 | ND | – |
| | | | | $(dI)_n \cdot (rC)_n$ | <8 | ND | – |
| $(I)_n \cdot (C)_n$ and analogs | | | | $(rI)_n \cdot (dC)_n$ | <8 | ND | – |
| $(I)_n \cdot (C)_n$ | 8000[e] | ++ | ++ | | | | |
| $(I)_n \cdot (br^5C)_n$ | 4000 | ++ | – | Miscellaneous | | | |
| $(c^7I)_n \cdot (C)_n$ | 4000 | – | – | EMCV RNA | <8 | ND | ND |
| $(c^7I)_n \cdot (br^5C)_n$ | 400 | ++ | – | globin mRNA | <8 | ND | ND |
| $(I)_n \cdot (s^2C)_n$ | 120 | – | – | Qβ RNA | 800 | ND | ND |
| | | | | MS-2 RNA | 12 | ND | ND |
| | | | | R17 RNA | 20 | ND | ND |

[a] Results expressed as nmol 2–5A synthesized per liter per hour in a standard reaction mixture (100 μl) [11,14] for mouse enzyme adsorbed to 2′,5′-ADP Sepharose. Each nucleic acid was evaluated at a final concentration of $2 \times 10^{-4}$ $M_p$

[b] Assayed as described previously [15,16,23]. ++ refers to intense phosphorylation of protein $P_1$ equal to that induced by $(I)_n \cdot (C)_n$; + refers to definite phosphorylation compared to extracts containing no $(I)_n \cdot (C)_n$, but less intense than that induced by $(I)_n \cdot (C)_n$ itself; alternatively, + implies that at a concentration of $2 \times 10^{-5}$ $M_p$ the polynucleotide gave rise to just as intense a phosphorylation of protein $P_1$ as did $(I)_n \cdot (C)_n$ but did so less effectively at lower $(I)_n \cdot (C)_n$ concentrations ($2 \times 10^{-6}$ $M_p$, $2 \times 10^{-7}$ $M_p$). Buffer 1: 20 mM HEPES (pH 7.5), 10 mM KCl, 60 mM KOAc, 0.5 mM DTT, 25 mM $Mg(OAc)_2$. Buffer 2 (protein synthesis buffer): 30 mM HEPES (pH 7.5), 110 mM KCl, 7 mM β-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 10 mM creatine phosphate, 40 μM twenty amino acids minus leucine, 0.16 mg creatine kinase (163 units $mg^{-1}$). Each polynucleotide was evaluated initially at $2 \times 10^{-5}$ $M_p$

[c] Includes $(A)_n$, $(U)_n$, $(I)_n$, $(C)_n$, $(X)_n$, $(s^2C)_n$, $(br^5C)_n$, $(dUz)_n$ and $(m^7G)_n$

[d] Samples of $(I)_n \cdot (C)_n$ were prepared by annealling $(I)_n$ and $(C)_n$ of varying molecular weights. The molecular size (in Svedberg units) of each individual strand is indicated in parentheses immediately after the abbreviation for the polynucleotide: thus, I(12.5)C(13.2) refers to dsRNA constituted from $(I)_n$ of $s_{20,w} = 12.5$ and $(C)_n$ of $s_{20,w} = 13.2$ S

[e] range: 2000–10 000

## 2. Material and methods

Cell-free extracts of interferon-treated or untreated mouse L cells and human Namalwa lymphoblastoid cells were prepared as described previously [9,11,12]. Sources and preparation of synthetic and naturally occurring polynucleotides were the same as outlined before [9,13]. Assays for the 2–5A synthetase and 2–5A itself as well as the protein $P_1$ kinase have been detailed elsewhere [11,12,14–16].

## 3. Results and discussion

We have studied the nucleic acid activation of human or mouse 2–5A synthetase under four separate assay conditions: (i) after adsorption to 2′,5′-ADP Sepharose; (ii) after adsorption to nucleic acids covalently linked to agarose; (iii) in solution under conditions of protein synthesis; and (iv) in solution under conditions idealized for activity of the human enzyme. The results of such experiments are presented in tables 1 and 2.

For evaluation of the ability of nucleic acids to activate the protein $P_1$ kinase, two assay systems were employed and both used extracts of interferon-treated mouse L cells (table 1). Since mouse L cells contain a phosphoprotein phosphatase activity which can be modified by nucleic acids as well as by the conditions of the assay [16], it is necessary to study the phosphorylation of protein $P_1$ under both conditions idealized for kinase activity and under conditions of protein synthesis.

The following conclusions can be drawn from the data of tables 1 and 2.

(1) Activation of the 2–5A synthetase and protein $P_1$ kinase are dependent on the double-stranded nature of the nucleic acid. One apparent exception is Qβ RNA which is nominally single-stranded, but activates the synthetase; however, Qβ RNA inhibits protein synthesis in reticulocyte lysates [17] and has been shown to contain up to 2% dsRNA [18]. This may be associated with a minor component of Qβ RNA which is or behaves like dsRNA [17,18]. The fact that R17, MS2, globin and EMCV RNA do not activate the synthetase

Table 2
Activation of 2–5A Synthetase by Selected Polynucleotides Under Different Assay Conditions

| Polynucleotide | L-cell enzyme[a] | | Namalwa cell enzyme[b] | |
|---|---|---|---|---|
| | Sepharose-bound[c] polynucleotide | Protein synthesis[d] conditions | Solution assay 25 mM $Mg^{2+}$ | 2′,5′-ADP-Sepharose-bound, 25 mM $Mg^{2+}$ |
| $(I)_n \cdot (C)_n$ | 2500 | ND | 33 | 2 |
| $(I)_n \cdot (br^5C)_n$ | 2500 | ND | 0.33 | 0.14 |
| $(I)_n \cdot (s^2C)_n$ | <40 | ND | 0.44 | ND |
| $(A)_n \cdot (U)_n$ | ND | <<4 | 6.6 | 0.02 |
| I(12.5) · C(3)[e] | ND | 40 000 | ND | ND |
| I(2.5) · C(13.2) | ND | 20 | ND | ND |
| I(12.5) · C(13.2) | ND | 40 000 | ND | ND |
| I(2.5) · C(3) | ND | 20 | ND | ND |

[a] The formation of 2–5A is given as nmol $l^{-1}$ $h^{-1}$ in a standard reaction mixture

[b] The formation of 2–5A is given as nmol $mg^{-1}$ protein $h^{-1}$ in a reaction mixture of 100 μl volume

[c] $(C)_n$, $(br^5C)_n$, or $(s^2C)_n$ was annealled to AGpoly(I)™ (PL Biochemicals) in Tris-buffered saline and then 100 μl of cell sap from interferon-treated cells was adsorbed to the thoroughly washed resin for a periods of 12–16 h, after which the resin was exhaustively washed with buffer and further processed as described for 2′,5′-ADP-Sepharose-bound enzyme [11,14]

[d] Conditions were identical to these described for protein synthesis in [9], except that interferon-treated L cell S10s had not been treated with micrococcal nuclease or preincubated

[e] These differently sized preparations of $(I)_n \cdot (C)_n$ were constituted from homopolymers of varying molecular size (see table 1)

Table 3
Comparative Potencies of Various RNAs for the Activation of Mouse L Cell 2–5A Synthetase Adsorbed to 2′,5′-ADP Sepharose

| Polynucleotide | $M_{act}$[a] |
|---|---|
| *P. chrysogenum* dsRNA | $1.5 \times 10^{-7}\ M_p$ |
| bacteriophage dsRNA | $3 \times 10^{-7}\ M_p$ |
| killer yeast dsRNA | $9 \times 10^{-7}\ M_p$ |
| $(I)_n \cdot (C)_n$ | $5 \times 10^{-7}\ M_p$ |
| $(c^7I)_n \cdot (C)_n$ | $5.5 \times 10^{-6}\ M_p$ |
| $(I)_n \cdot (br^5C)_n$ | $1.8 \times 10^{-5}\ M_p$ |
| $(c^7I)_n \cdot (br^5C)_n$ | $1.4 \times 10^{-4}\ M_p$ |
| bacteriophage Qβ RNA | $4 \times 10^{-4}\ M_p$ |

[a] Represents a comparison of the ability to various nucleic acids to activate the synthetase and is defined as the molar concentration of RNA required to produce sufficient 2–5A to effect, after a 10 000-fold dilution, a 33% reduction in EMCV RNA-stimulated protein synthesis in cell free extracts of interferon-treated mouse L cells. The smaller the $M_{act}$, the more potent the nucleic acid as a synthetase activator

even at quite high concentrations suggests either that the short dsRNA hairpin structures occurring in the secondary structure of these RNAs cannot activate the synthetase because they are too short or that they may not be conveniently accessible to the synthetase. The alternating copolymer $(A–U)_n$ leads to substantial 2–5A synthesis, implying that a hairpin structure of sufficient length is capable of activating the synthetase.

(2) The presence of a 2′-hydroxyl group in both strands of the nucleic acid seems critical to its ability to activate 2–5A synthetase. The presence of a 2′-hydroxyl group is not absolutely required for activation of the protein $P_1$ kinase since $(A)_n \cdot (dUfl)_n$ and $(I)_n \cdot (Cm)_n$ could both activate the kinase, albeit not as well as $(I)_n \cdot (C)_n$ itself.

(3) Although quite a number of nucleic acids viz., [$(I)_n \cdot (C)_n$, $(A–U)_n$, *P. chrysogenum* dsRNA, killer yeast dsRNA, f2 bacteriophage dsRNA, $(I)_n \cdot (br^5C)_n$, $(c^7I)_n \cdot (C)_n$, $(c^7I) \cdot (br^5C)_n$, $(I)_n \cdot (s^2C)_n$, $(A)_n \cdot (U)_n$, $(c^7A)_n \cdot (U)_n$, $(c^7A)_n \cdot (rT)_n$ and $(c^7A)_n \cdot (br^5U)_n$] are capable of activating the 2–5A synthetase, only a few are comparable in potency to $(I)_n \cdot (C)_n$ (tables 1 and 3).

(4) When synthetase activity is analyzed under considerably different assay conditions (table 2), the following points emerge:

(i) Under all conditions, $(I)_n \cdot (C)_n$ is the most potent activator (with the possible exception of the natural dsRNAs).

(ii) When analyzed by adsorption of Namalwa enzyme to 2′,5′-ADP Sepharose, $(I)_n \cdot (br^5C)_n$ is a more potent synthetase activator than is $(A)_n \cdot (U)_n$; however, when the assay is conducted in solution at high $Mg^{2+}$ concentration, $(A)_n \cdot (U)_n$ is a more potent activator than is $(I)_n \cdot (br^5C)_n$. In other words, high $Mg^{2+}$ concentrations dramatically increase the potency of $(A)_n \cdot (U)_n$ as a synthetase activator but does not affect substantially the relative potency of other activators.

Previous studies have probed the possible relationship between inhibition of protein synthesis by dsRNA in extracts of interferon-treated cells or rabbit reticulocyte lysates and interferon induction by dsRNA [9,10]. These studies concluded that, although there existed definite similarities in the structural features required for interferon induction and for protein synthesis inhibition, there were sufficient exceptions to deny any simple relationship. Studies with the enzyme systems that presumably lead to protein synthesis inhibition by dsRNA, namely, the 2–5A synthetase and the protein $P_1$ kinase, have concluded that the similarity in certain requirements of molecular size and presence of 2′-hydroxyl groups suggest a relationship between synthetase activation and interferon induction [19,21]. The data presented in this paper suggest that there may be no simple relationship between interferon induction by polynucleotides and activation of either 2-5A synthetase or the protein $P_1$ kinase.

The polynucleotide $(I)_n \cdot (C)_n$, $(I)_n \cdot (br^5C)_n$, $(A)_n \cdot (rT)_n$, $(A)_n \cdot (U)_n$, $(c^7I)_n \cdot (C)_n$, $(c^7I)_n \cdot (br^5C)_n$, *P. chrysogenum* dsRNA, f2 bacteriophage dsRNA, killer yeast dsRNA, $(A–U)_n$ and $(I)_n \cdot (s^2C)_n$ are all good inducers of interferon in various cell cultures and in animals [2,13]; however, $(A)_n \cdot (rT)_n$ does not activate 2–5A synthetase at all, and $(A)_n \cdot (U)_n$ and $(I)_n \cdot (s^2C)_n$ are very poor activators. Conversely, $(c^7A)_n \cdot (U)_n$, $(c^7A)_n \cdot (rT)_n$ and $(c^7A)_n \cdot (br^5U)_n$ are not active as interferon inducers [2] but do activate the synthetase, albeit rather ineffectively. In addition,

$(dIz)_n \cdot (C)_n$, a 2′-modified polymer which induces interferon [21], is inactive as an activator of synthetase (P. F. Torrence, E. De Clercq and M. Ikehara, unpublished observations). Thus, no direct relationship between interferon induction and 2–5A synthetase activation can be implied.

It is also unlikely that any direct relationship exists between interferon induction and activation of the protein $P_1$ kinase for the following reasons:

(i) Three triple-helical nucleic acids, inactive as interferon-inducers, are good activators of the kinase.

(ii) The polynucleotides, $(I)_n \cdot (s^2C)_n$ and $(c^7I)_n \cdot (C)_n$, which are good interferon inducers, do not activate the kinase.

(iii) The 2′-modified polymers, $(A)_n \cdot (dUfl)_n$ and $(I)_n \cdot (Cm)_n$, which do not induce interferon, are activators of the protein $P_1$ kinase.

The data of table 2 illustrate the complexity of the 2–5A synthetase and sound a note of caution regarding general conclusions about the relationship of the 2–5A synthetase to various biological activities of dsRNAs. Depending on the nature of the enzyme, the concentration of divalent cations and whether the enzyme is bound to a matrix or free in solution, nucleic acids may be relatively active or inactive as activators; in addition, their relative potencies as activators may vary. Perhaps at least a part of this variation may be linked to the phosphodiesterase activity which degrades 2–5A [22].

A final point pertains to the relationship between inhibition of translation by dsRNA and the two dsRNA-dependent enzyme systems, the protein $P_1$ kinase and the 2–5A synthetase, believed to be responsible for mediating the effect of dsRNA. The triplexes $(A)_n \cdot (U)_n \cdot (X)_n$ and $(A)_n \cdot (U)_n \cdot (I)_n$ are effective inhibitors of protein synthesis in extracts of interferon-treated cells [9] and good activators of the protein $P_1$ kinase (table 1), but do not activate the 2–5A synthetase; in addition, $(A)_n \cdot (U)_n$ is a relatively poor activator of synthetase under most conditions (tables 1 and 2), but is quite effective as an activator of kinase and as an inhibitor of translation. The duplex, $(I)_n \cdot (s^2C)_n$, is a very poor synthetase activator and is inactive as a kinase activator; perhaps as a result it is also inactive as an inhibitor of protein synthesis. $(I)_n \cdot (C)_n$ is a potent activator of both kinase and synthetase, but recent evidence suggests that in extracts of interferon-treated L cells, the 2–5A system is primarily responsible for inhibition of translation [23]. Taken together, these data suggest that different nucleic acids or different dsRNAs might inhibit protein synthesis by different mechanisms.

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