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The 2-5A System: Modulation of Viral and Cellular Processes Through Acceleration of RNA Degradation

Mark R. Player and Paul F. Torrence*

SECTION ON BIOMEDICAL CHEMISTRY, LABORATORY OF MEDICINAL CHEMISTRY, BUILDING 8, ROOM B2A02,
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES,
NATIONAL INSTITUTES OF HEALTH, BETHESDA, MD 20892-0805, USA

ABSTRACT. The 2-5A system is an RNA degradation pathway that can be induced by the interferons (IFNs). Treatment of cells with IFN activates genes encoding several double-stranded RNA (dsRNA)-dependent synthetases. These enzymes generate 5'-triphosphorylated, 2',5'-phosphodiester-linked oligoadenylates (2-5A) from ATP. The effects of 2-5A in cells are transient since 2-5A is unstable in cells due to the activities of phosphodiesterase and phosphatase. 2-5A activates the endoribonuclease 2-5A-dependent RNase L, causing degradation of single-stranded RNA with moderate specificity. The human 2-5A-dependent RNase is an 83.5 kDa polypeptide that has little, if any, RNase activity, unless 2-5A is present. 2-5A binding to RNase L switches the enzyme from its off-state to its on-state. At least three 2',5'-linked oligoadenylates and a single 5'-phosphoryl group are required for maximal activation of the RNase. Even though the constitutive presence of 2-5A-dependent RNase is observed in nearly all mammalian cell types, cellular amounts of 2-5A-dependent mRNA and activity can increase after IFN treatment. One well-established role of the 2-5A system is as a host defense against some types of viruses. Since virus infection of cells results in the production and secretion of IFNs, and since dsRNA is both a frequent product of virus infection and an activator of 2-5A synthesis, the replication of encephalomyocarditis virus, which produces dsRNA during its life cycle, is greatly suppressed in IFN-treated cells as a direct result of RNA decay by the activated 2-5A-dependent RNase. This review covers the organic chemistry, enzymology, and molecular biology of 2-5A and its associated enzymes. Additional possible biological roles of the 2-5A system, such as in cell growth and differentiation, human immunodeficiency virus replication, heat shock, atherosclerotic plaque, pathogenesis of Type I diabetes, and apoptosis, are presented. PHARMACOL. THER. 78(2):55–113, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Double-stranded RNA, 2-5A, interferons, ribonuclease L, virus, host defense.

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ABBREVIATIONS. 2-5A, (pp)p5'A2'(p5'A2')_n; AIDS, acquired immunodeficiency syndrome; ANK, ankyrin; bTTP-1, bovine trophoblastic protein; cAMP, cyclic AMP; CD, circular dichroism; CFS, chronic fatigue syndrome; CML, chronic myelogenous leukemia; dsRNA, double-stranded RNA; EMCV, encephalomyocarditis virus; HHV, human herpes virus; HIV, human immunodeficiency virus; hnRNA, heterogeneous nuclear RNA; HPLC, high performance liquid chromatography; HSIF, heat shock-induced factor; hsp, heat shock protein; IDDM, insulin-dependent diabetes mellitus; IFN, interferon; IL, interleukin; ISRE, interferon-stimulatable response element; LTR, long terminal repeat; MSV, murine sarcoma virus; NGF, nerve growth factor; ORF, open reading frame; oTP-1, ovine trophoblastic protein; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cell; PDGF, platelet-derived growth factor; PKC, protein kinase C; PKR, p68 dsRNA-dependent protein kinase; PMBC, polymorphonuclear blood cell; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; rRNA, ribosomal RNA; RSV, respiratory syncytial virus; RT-PCR, reverse transcription-coupled polymerase chain reaction methodology; ssRNA, single-stranded RNA; TAR, *trans*-acting response element; TGF, transforming growth factor; TLC, thin-layer chromatography; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; TRI, tubular reticular inclusions; VSV, vesicular stomatitis virus.

1. INTRODUCTION

Sundry low molecular mass nucleotides, in addition to the cyclic mononucleotides cyclic AMP (cAMP) and cyclic GMP, have been described and pursued for their fascinating biological properties. For instance, guanosine 5'-diphosphate 3'-diphosphate, a "magic spot" nucleotide, has been established as an intracellular messenger that influences the transcription rate of various genes and as the mediator of the stringent response in *Escherichia coli* (Gallant, 1979). Diadenosine 5',5'''-P¹,P⁴-tetrphosphate, originally described by Zamecnik *et al.* (1966) in the back reaction of lysyl-tRNA synthetase, is an established competitive inhibitor of platelet aggregation, and its analogues have been pursued as antithrombotic agents (Chan *et al.*, 1997). Nicotinic acid adenine dinucleotide phosphate, 2'-phospho-cyclic ADP-ribose, and cyclic ADP-ribose have been found to regulate calcium signaling (Genazzani and Galione, 1997).

This review encompasses a unique group of low molecular mass molecules referred to as 2-5A (Fig. 1), represented as (pp)p5'A2'(p5'A2')_n. Interest in this field is attested to by the volume of publications dealing with 2-5A: in excess of 500. The groundwork for the discovery of 2-5A was laid through the efforts of a variety of laboratories (Table 1). That work led to the now classical investigations of Kerr and Brown (1978) that described the exact chemical nature of 2-5A. This paper (Kerr and Brown, 1978) should be required reading of students entering the field of nucleic acid chemistry and biochemistry because of its elegant application of basic chemical and enzymatic principles to structure elucidation. Although the chemical structure of 2-5A was described nearly two decades ago, it has only been recently that the key enzyme in 2-5A action, the RNase L, has been cloned and finally purified to homogeneity (Table 1). Much has been accomplished in this area since the last

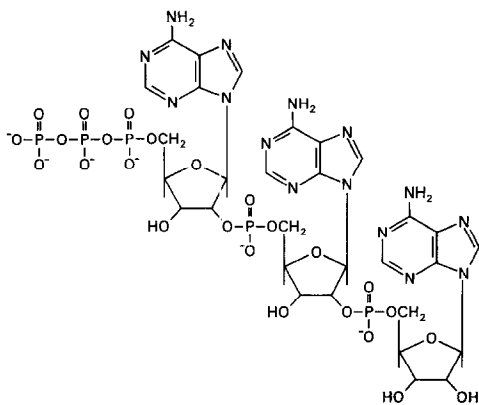


FIGURE 1. Structure of 2-5A trimer 5'-O-triphosphoryladenyl(2'→5')adenyl(2'→5')adenosine.

time one of us co-authored a review of the 2-5A system (Johnston and Torrence, 1984). Then it was possible to review all aspects of double-stranded RNA (dsRNA) (Lengyel, 1987) in the interferon (IFN)-induced inhibition of viral replication. The present effort is an attempt to consolidate the salient findings and features of the 2-5A system alone (Fig. 2).

2. SYNTHESIS OF 2-5A AND ITS ANALOGUES

The unique nature of 2-5A (Fig. 1) was the impetus for a host of publications that detailed virtually every conceivable chemical approach to such a molecule (Table 2). Quite a number of reported syntheses stopped short of providing the key 5'-mono-, di-, and triphosphate moieties required for biological activity (Table 2). However, these latter key transformations can be effected on an appropriately protected oligonucleotide substrate using a variety of approaches (Table 3). The application of these chemistries and others has led to a considerable formulary of 2-5A analogues with modifications to the ribose moieties, the bases, the phosphate backbone, and the 5'-mono/polyphosphate residue (Table 4). In addition, a respectable number of ligands and other alterations have been added to the termini of the molecule (Table 4). 2-5A has also been ligated to normal linkage (3'→5') nucleic acids (Torrence *et al.*, 1993).

3. 2-5A SYNTHETASE, A DOUBLE-STRANDED RNA-REGULATED NUCLEOTIDYL TRANSFERASE

3.1. Occurrence

2-5A synthetase activity has been demonstrated to be present in a large variety of mammalian species, including that of the mouse, rabbit, dog, guinea pig, hamster, pig, monkey, cow, and humans. It has also been detected in viruses, reptiles, and several avian species, including chickens (Table 5). *Drosophila*, amphibians, yeast, slime molds, and bacteria, however, have not been shown to possess 2-5A synthetase activity (Stark *et al.*, 1979; Cayley *et al.*, 1982b).

However, 2-5A synthetase recently has been reported to be present in the marine sponge *Geodia cydonium*, based on poly(I).poly(C) binding, 2-5A formation, and Western blotting with an antibody to rat 43 kDa synthetase (Kuusksalu *et al.*, 1995).

Although 2-5A synthetase has not been identified in plants (Cayley *et al.*, 1982b), virus resistance has been achieved by cloning a rat cDNA for 2-5A synthetase followed by transformation of tobacco and potato (Truve *et al.*, 1993, 1994a).

2-5A synthetase activity has been found in a large number of human cell types, but basal levels can vary greatly. The activity has been found in both nuclei and cytoplasm (Nilsen *et al.*, 1982c). Human diploid fibroblasts (MRC5) contain very low levels of 2-5A synthetase (Stark *et al.*, 1979), while Namalva (Johnston *et al.*, 1980b), HeLa (Silverman *et al.*, 1982), and K/BALB cells (Meurs *et al.*, 1986) express high levels in the absence of IFN induction. The enzyme occurs in high levels in human peripheral blood cells (Bruchelt *et al.*, 1992) and human umbilical cord blood mononuclear cells (Williams and Read, 1981). *Homo sapiens* males have been found to have an 8-fold higher concentration of the enzyme in their polymorphonuclear blood cells (PMBCs) (Miele *et al.*, 1992). Monocytes separated by density gradient centrifugation had 3- to 5-fold greater 2-5A synthetase levels than did lymphocytes, while B lymphocytes separated from T lymphocytes by fluorescence-activated cell sorting had a 2-fold higher level of the enzyme (Witt *et al.*, 1990; Buffet-Janvresse *et al.*, 1986). 2-5A synthetase is also present in the serum and plasma of mice (Krishnan and Baglioni, 1980a) and humans (Bruchelt *et al.*, 1992; Rusconi *et al.*, 1994). Its clinical usefulness for monitoring IFN therapy will be discussed in the following section.

3.2. Induction

The most important inducer of 2-5A synthetase is IFN. The degree and time course of induction depends on the dose and type of IFN, length of exposure, and cell type. The extent of induction observed has been generally several-fold to several hundred-fold, although a remarkable 10⁴-fold induction has been reported in chick embryo cells (Ball, 1979). It should be noted that many of these induction studies have been based on enzyme activity levels, as opposed to measurement of protein. A 1:1 linear relationship has not been established.

A number of investigators have demonstrated differential induction with Type I versus Type II IFNs. Wallach *et al.* (1982) found lower levels of 2-5A synthetase in human SV80 fibroblasts and Ramos cells after treatment with IFN- γ compared with IFN- β . In mouse L929 cells, however, 2-5A synthetase levels were similar after IFN- β or - γ treatment (Hovanessian *et al.*, 1980). IFN- γ has been shown to induce lower 2-5A synthetase levels than IFN- β in the monocytic line U937, the myelocytic line HL-60, and human peritoneal macrophages (Witt *et al.*, 1990), as well as *in vivo* in

TABLE 1. Signal Discoveries in the Scientific Evolution of the 2-5A System

Discovery	Reference
Discovery of IFN.	Isaacs and Lindemann, 1957
Inhibition of protein synthesis in IFN-treated virus-infected cells.	Joklik and Merigan, 1966
Detection of dsRNA in extracts of vaccinia virus-infected cells.	Duesberg and Colby, 1969
Cell toxicity in response to combined treatment with both IFN and dsRNA.	De Clercq <i>et al.</i> , 1973
The ribosome as a site of IFN action: inability of ribosomes from IFN-treated cells to translate viral RNA. Concept of translation inhibitory protein.	Marcus and Salb, 1966
Cell-free extracts of IFN-treated cells were deficient in their ability to translate Mengovirus RNA, but not poly(U) or tobacco mosaic virus RNA.	Carter and Levy, 1968
No effect of IFN treatment of cells discerned in cell-free extracts until such extracts were preincubated. Responsible factor was ribosome-associated, but not species-specific.	Samuel and Joklik, 1974; Samuel and Ferris, 1977
Reduced ability of IFN-treated chick cell ribosomes to translate EMCV RNA.	Kerr, 1971
Extracts from IFN-treated and vaccinia virus-infected cells showed reduced ability to translate both EMCV RNA and globin mRNA.	Friedman <i>et al.</i> , 1972a,b; Kerr <i>et al.</i> , 1973
Extracts of IFN-treated mouse L-cells showed greatly reduced ability to translate Mengovirus RNA and globin RNA, but ability to translate poly(U) was only marginally affected.	Falcoff <i>et al.</i> , 1973
In coupled transcription-translation system from primary chick embryo cells, there was no effect of IFN on transcription, but various mRNAs were inhibited from translation in the absence of dsRNA.	Ball and White, 1978a,b
IFN-inducible ribosome-associated translational inhibitor in ascites tumor cells.	Gupta <i>et al.</i> , 1973, 1974a,b
Extracts from IFN-treated and virus-infected mouse L-cells showed reduction in amount of EMCV RNA-specified products and also decrease in molecular weight of products.	Kerr <i>et al.</i> , 1974b
Extracts from IFN-treated L-cells showed exquisite sensitivity of translational inhibition by dsRNA. The addition of dsRNA to the cell extracts obviated the requirement for virus infection demonstrated in the study by Friedman <i>et al.</i> (1972a,b).	Kerr <i>et al.</i> , 1974a
IFN-inducible dsRNA-mediated inhibition of protein synthesis confirmed in cell-free extracts from mouse, human, and chick cells.	Shailla <i>et al.</i> , 1977; Ball and White, 1978a,b
Addition of dsRNA to extracts of IFN-treated cells accelerated the rate of degradation of mRNA.	Brown <i>et al.</i> , 1976; Kerr <i>et al.</i> , 1976; Ball and White, 1978b; Clemens and Vaquero, 1978; Lewis <i>et al.</i> , 1978
Both dsRNA and ATP were required for the dsRNA-stimulated RNA degradation.	Ratner <i>et al.</i> , 1978; Roberts <i>et al.</i> , 1976; Sen <i>et al.</i> , 1976
A low-molecular weight inhibitor of translation was generated when extracts of IFN-treated mouse L-cells were incubated with dsRNA and ATP.	Roberts <i>et al.</i> , 1976
The above low-molecular weight inhibitor could be synthesized on a column of poly(I)-poly(C)-agarose to which the low-molecular weight inhibitor synthetic activity of IFN-treated cells had been adsorbed.	Hovanessian <i>et al.</i> , 1977
Identification of low-molecular weight inhibitor as ppp5' A2' p5' A2' p5' A, called 2-5A.	Kerr and Brown, 1978
Verification of generation of 2-5A in other systems.	Ball and White, 1978c; Hovanessian and Kerr, 1979; Minks <i>et al.</i> , 1979a
Verification of structure of 2-5A by chemical synthesis.	den Hartog <i>et al.</i> , 1979, 1981; Engels and Krahmer, 1979; Ikehara <i>et al.</i> , 1979, 1981; Jones and Reese, 1979; Markham <i>et al.</i> , 1979; Ogilvie and Theriault, 1979; Sawai <i>et al.</i> , 1979; see also Table 2
Activation of nuclease activity by 2-5A.	Clemens and Williams, 1978; Farrell <i>et al.</i> , 1978; Ball and White, 1979b; Slattery <i>et al.</i> , 1979; Eppstein <i>et al.</i> , 1979; Williams <i>et al.</i> , 1978; Schmidt <i>et al.</i> , 1978; Chernajovsky <i>et al.</i> , 1979; Baglioni <i>et al.</i> , 1978
Accumulation of 2-5A in IFN-treated virus-infected cells.	Williams <i>et al.</i> , 1979b; Knight <i>et al.</i> , 1980
Evidence for localized activation of 2-5A system in cell extracts.	Nilsen and Baglioni, 1979
Increased concentrations of 2-5A in organs of EMCV-infected mice.	Hearl and Johnston, 1987
2',5'-Phosphodiesterase purification and characterization.	Williams <i>et al.</i> , 1978; Schmidt <i>et al.</i> , 1979; Minks <i>et al.</i> , 1979a,b; Johnston and Hearl, 1987
Prevention of 2-5A action by 5'-monophosphate of 2-5A: p5' A2' p5' A2' p5' A.	Torrence <i>et al.</i> , 1981a
Prevention of anti-EMCV activity of IFN by 2-5A analogue acting as a competitive inhibitor of 2-5A.	Warling <i>et al.</i> , 1985
RNase L-characteristic cleavage products of rRNA detected in IFN-treated virus-infected cells.	Wreschner <i>et al.</i> , 1981a; Silverman <i>et al.</i> , 1982, 1983

(continued)

TABLE 1. Continued

Discovery	Reference
Cleavage specificity for RNase L established.	Wreschner <i>et al.</i> , 1981b; Floyd-Smith <i>et al.</i> , 1981
Identification of four different forms of 2-5A synthetase in human cells.	Chebath <i>et al.</i> , 1987a
Purification of 2-5A synthetase.	Ball and White, 1979a; Wells <i>et al.</i> , 1984; Dougherty <i>et al.</i> , 1980; Ball, 1980; Yang <i>et al.</i> , 1981; Marie <i>et al.</i> , 1989; Ilson <i>et al.</i> , 1986
Dominant-negative mutant of RNase L, that bound 2-5A efficiently, but lacked nuclease activity, when expressed in murine cells, prevented the anti-EMCV effect of IFN.	Hassel <i>et al.</i> , 1993
Activated 40 kDa form of 2-5A synthetase found bound to viral RNA in IFN-treated EMCV-infected HeLa cells.	Gribaudo <i>et al.</i> , 1991
Cloning of human and mouse RNase L.	Zhou <i>et al.</i> , 1993; Hassel <i>et al.</i> , 1993
Introduction of 2-5A-antisense.	Torrence <i>et al.</i> , 1993, 1994

total peripheral blood mononuclear cells (PBMCs) (Witt *et al.*, 1989). IFN- α and - β have been shown to bind to a different class of cell surface receptor (Type I) than IFN- γ (Type II) (Branca and Baglioni, 1981). Since the type of cell determines both the respective receptor concentrations and their K_d , and transcriptional regulation of 2-5A synthetase has been closely tied to receptor occupancy (Hannigan and Williams, 1986), it is not surprising that induction events would differ between these two classes of IFNs.

When HeLa cells were treated with IFN- β , after a 3-hr lag, there was a rapid rise in 2-5A synthetase levels, which peaked and remained constant at about 24 hr (Baglioni *et al.*, 1979). This reflected *de novo* protein synthesis. The time course of induction of 2-5A synthetase with IFN- γ has been found to be slower than that found with Type I IFNs in HeLa cells (Baglioni and Maroney, 1980). A number of studies have specifically examined the kinetics of *in vivo* induction of 2-5A synthetase. Lodemann *et al.* (1985) found that after a dose of 5×10^4 U/kg of IFN- α , 2-5A synthetase measured in PBMCs peaked at 6 hr, and that the i.v. route was not superior to i.m. injection. In another trial of IFN- α , the range of induction of 2-5A synthetase was found to be 2.3- to 5.7-fold; however, the extent and duration of the 2-5A synthetase response was a function of only the dose and not the route or number of injections (Merritt *et al.*, 1992). After a dose of $1-5 \times 10^6$ U of IFN- β s.c., 2-5A synthetase

levels peaked at 24-48 hr in PBMC lysates and 48-72 hr in serum. A dose of $2-4 \times 10^6$ U of IFN- γ caused a smaller increase in 2-5A synthetase (Bruchelt *et al.*, 1992). A trial of an IFN- β/γ combination together as a continuous infusion found dose-limiting toxicity at 3×10^6 U of IFN- β and 200 mg of IFN- γ , consisting of refractory headache, fever, and hepatic toxicity (Schiller *et al.*, 1990).

It also has been well established that Type I IFNs induce transcription of the 2-5A synthetase gene in the presence of cycloheximide, unlike Type II IFNs, which generally do not (Dianzani *et al.*, 1980; Faltynek *et al.*, 1985; Gribaudo *et al.*, 1989). This implied that 2-5A synthetase was induced as a primary response to treatment of cells with Type I IFNs since no new protein synthesis was required; however, new protein synthesis was required for induction of 2-5A synthetase by IFN- γ . There is some early evidence for superinduction of 2-5A synthetase. When chick embryo fibroblasts were stimulated with homologous IFN, the 2-5A synthetase level peaked at about 9 hr. If IFN was then removed, the addition of cycloheximide could delay the decay (West and Ball, 1982) or restore (Lab *et al.*, 1982) 2-5A synthetase activity.

Four isoforms (p40, p44, p69, p100) of IFN have been described (Chebath *et al.*, 1987a; Hovanessian *et al.*, 1987). The various isoforms are induced differentially with a given IFN subtype. In Hs294t melanoma cells, after 48 hr of

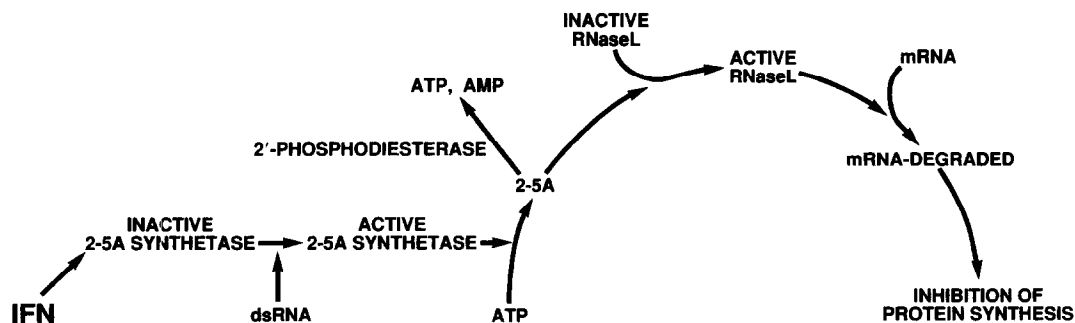


FIGURE 2. A schematic representation of the principal components of the 2-5A system.

TABLE 2. Synthetic Approaches to 2-5A and Related Molecules

Investigators	Condensing or activating reagents comments	Synthons	Product(s)
Ikehara <i>et al.</i> , 1979, 1981	POCl ₃ in PO(OEt) ₃	3'-O-(<i>o</i> -nitrobenzoyl)-N ⁶ -benzoyladenine 5'-phosphate	(p5'/A2') _n n = 2-5
Ikehara <i>et al.</i> , 1981	Mesitylene sulfonyl triazolide	5'-O-MMT-3'-O-(<i>o</i> -nitrobenzoyl)-N ⁶ -benzoyladenine 2'-O- <i>p</i> -chlorophenylphosphate and 5'-O-phosphorodiamidido-3'-O-(<i>o</i> -nitrobenzoyl)-N ⁶ -benzoyladenine	p5'/A2', p5'/A p5'/A2', p5'/A2', p5'/A
Shimidzu <i>et al.</i> , 1984	Tris-(imidazol-1-yl) phosphine. N protection not necessary	Adenosine or N ⁶ -benzoyladenine	(Ap) _n A, n = 1-4
Hayakawa <i>et al.</i> , 1985a,b	Triester approach: reaction of phosphorochloridate with Mg alkoxide of protected nucleoside. No N protection	2',3'-Di-O-TBDMS-adenosine and 5'-MMT-3'-O-TBDMS-adenosine 2'-O- <i>o</i> -chlorophenyl- <i>p</i> -nitrophenyl phosphate	A2', p5'/A2', p5'/N N = A, U, C, G
Chattopadhyaya, 1980	1-Mesitylenesulfonyl-3-nitro-1,2,4-triazole	5'-Pixyl-3'-O-(2-dibromomethylbenzoyl)-N-benzoyladenine 2'-O-chlorophenyl-phosphate and N ⁶ , N ⁶ , O ² , 3'-tetra-benzoyl adenosine	A2', p5'/A2', p5'/A
Jones and Reese, 1979	1-Mesitylenesulfonyl-3-nitro-1,2,4-triazole	5'-O- <i>p</i> -chlorophenoxyacetyl-3'-O-methoxytetrahydropyranyl-N ⁶ -benzoyl adenosine 2'- <i>p</i> -nitrophenylphosphate and 2',3'-O-methoxymethylene-N ⁶ -benzoyladenine	A2', p5'/A2', 5p'/A and 5'-triphosphate
Charubala and Pfeleiderer, 1980a	Tri-isopropylbenzene sulfonyl nitrotriazolide	5'-MMT-3'-O-TBDMS-N ⁶ -benzoyladenine-3'-O-(<i>o</i> -chlorophenyl)phosphate and 3'-TBDMS-N ⁶ -benzoyladenine-3'-O-(<i>o</i> -chlorophenyl) phosphate or N ⁶ , N ⁶ , O ² , O ³ '-tetra-benzoyladenine	A2', p5'/A2', p5'/A
Charubala and Pfeleiderer, 1982	Quinoline 8-sulfonyl chloride and 3-nitro-q 1,2,4-triazole	5'-MMT-3'-TBDMS-inosine-2'-(<i>β</i> -nitrophenylethyl) phosphate and 3'-O-TBDMS-2'-(2,5-dichlorophenyl <i>p</i> -nitrophenylethyl)phosphate or 2',3'-TBDMS-adenosine of 2',3'-di-O-benzoyladenine	I2', p5'/I2', p5'/I I2', p5'/I2', p5'/I2', p5'/I
Engels and Kraemer, 1979	Triester approach: tri-isopropylbenzene sulfonylnitroimidazolide	5'-MMT-3'-O-N ⁶ -dibenzoyl-3'-tribromoethyl phosphate and 2',3',N ⁶ -tribenzoyladenine	A2', p5'/A2', p5'/A
Cioeli <i>et al.</i> , 1981	1-Mesitylenesulfonyl-3-nitro-1,2,4-triazole; use of tetraisopropyl-disiloxane-1,1,3,3-diyl protecting group	3',5'-O-TIPDSi-N ⁶ -benzoyladenine 2'-(<i>o</i> -chlorophenyl)phosphate and 2',3'-di-O-acetyl-N ⁶ -benzoyladenine	A2', p5'/A2', p5'/A

den Hartog <i>et al.</i> , 1979, 1981	Triester approach: tri- isopropylbenzene sulfonylnitroimidazolide	3'-O-(4-methoxytetrahydropyran-4-yl)-N ⁶ - <i>p</i> - anisoyladenine 5'-O-[2,2,2-tribromoethyl]- morpholinophosphate 2'-O-(2-chlorophenyl- phosphate and 3'-O-(4-methoxytetrahydropyran- 4-yl)-N ⁶ - <i>p</i> -anisoyladenine	(pp)p5' A2' p5' A2' p5' A, also 3'-O-methyl analogues
Sawai <i>et al.</i> , 1979, 1981 Imai and Torrence, 1985	Pb ²⁺ catalysis Pb ²⁺ catalysis	Adenosine 5'-phosphorimidazolide Nucleoside 5'-phosphorimidazolides and nucleoside 5'-phosphoromorpholides	(pp)p5' A2' p5' A2' p5' A (pp)p5' N2' p5' N2' p5' N N = A or I A2' p5' A2' p5' A
Ogilvie and Theriault, 1979; Imai and Torrence, 1981a Markham <i>et al.</i> , 1979	2,2,2-Trichloroethylphosphoro- dichloridite	5'-MMT-3'-O-TBDMS-adenosine and 2',3'-di-O- TBDMS-adenosine	A2' p5' A2' p5' A2' p
Karpeisky <i>et al.</i> , 1982	Tri-isopropylbenzene sulfonyl chloride: solid phase Tri-isopropylbenzene sulfonyl chloride	5'-MMT-3'-O-benzoyl-N ⁶ -benzoyl adenosine 2'- monophosphate 5'-MMT-3'-O- <i>p</i> -methylbenzoyl-N ⁶ -benzoyladenine 2'-(2-cyanoethyl)-phosphate and 2',3'-di-O-acetyl- N ⁶ -benzoyladenine	A2' p5' A2' p5' A A2' p5' A2' p5' A
Charubala <i>et al.</i> , 1981	Tri-isopropylbenzene sulfonyl chloride	5'-MMT-3'-O-TBDMS-N ⁶ -benzoyl-adenosine 2'-[2- (<i>p</i> -nitrophenyl)ethyl] phosphate and 2',3'-di-O- TBDMS-N-benzoyladenine	A2' p5' A2' p5' A
Takaku and Ueda, 1983	1-(Quinoly)sulfonyl)-1H- tetrazole	2',3',N ⁶ -tribenzoyladenine and 5'-DMT-N ⁶ ,3'-O- dibenzoyladenine 2'-(5-chloro-8-quinolyl) phosphate	A2' p5' A2' p5' A
Kvasnyuk <i>et al.</i> , 1987	Triester approach: 2,4,6-tri- isopropyl-benzene tetrazolide	N ⁶ , N ⁶ , 2',3'-tetrabenzoyladenine and N ⁶ ,3'- dibenzoyl-5'-O-MMT-adenosine 2'-[2-(4- nitrophenyl)-ethyl]-phosphate	A2' p5' A2' p5' A

DMT, dimethoxytrityl; MMT, *p*-monomethoxytrityl; TBDMS, *t*-butyl-dimethylsilyl; TIPDSi, 1,1,3,3-tetraisopropylidisiloxane-1,3-yl.

TABLE 3. Chemical Approaches to 2',5'-Oligonucleotide 5'-Mono-, Di-, and Triphosphates

Investigators	Strategy and reagents	Comments
den Hartog <i>et al.</i> , 1981	2,2,2-Tribromoethyl phosphoromorpholinochloridate used to introduce 5'-phosphate in monomeric synthons followed by Zn deblocking of the tribromoethyl moiety and conversion of the resultant 5'-phosphoromorpholidate to monophosphate with acid hydrolysis, or to 5'-diphosphate with tri- <i>n</i> -butylammonium phosphate, or to 5'-triphosphate with bis(tri- <i>n</i> -butylammonium)diphosphate.	This approach is advantageous as the critical 5'-phosphate is incorporated in masked form during chain extension. There is no need for a separate 5'-phosphorylation.
Ikehara <i>et al.</i> , 1981	Oligomerization of 3'-O-(<i>o</i> -nitrobenzoyl)-N ⁶ -benzoyl-adenosine gave the 5'-phosphate directly. The 5'-triphosphate was obtained through the intermediate phosphoroimidazolidate.	As above, the need for separate 5'-monophosphorylation is obviated by incorporation of final 5'-monophosphate into synthetic procedure.
Jones and Reese, 1979	Reaction of fully protected (except 5'-OH) trinucleotide with O-2,4-dichlorophenyl S-methylphosphorochloridothioate to yield, after deblocking, a 5'-S-methylphosphorothiate that was reacted with excess tetra(tri- <i>n</i> -butylammonium)-pyrophosphate.	Results in mixture of 5'-mono-, 5'-di-, and 5'-triphosphates.
Sawai <i>et al.</i> , 1979	Pb ²⁺ -catalyzed polymerization reaction product was 5'-monophosphate, which was converted to phosphoroimidazolidate and then to 5'-triphosphate with bis(tri- <i>n</i> -butylammonium)diphosphate.	
Imai and Torrence, 1985	Pb ²⁺ -catalyzed ligation of a nucleoside 5'-phosphoromorpholidate with a mononucleoside or dinucleotide 5'-phosphoroimidazolidate provided a 2',5'-linked oligonucleotide terminating in a 5'-phosphoroimidazolidate that could be converted into 5'-monophosphate through acid hydrolysis, or to 5'-di or 5'-triphosphate by reaction either with bis(tri- <i>n</i> -butylammonium) phosphate or with bis(tri- <i>n</i> -butylammonium)diphosphate, respectively.	The 5'-phosphoromorpholidate serves both as protecting group during 2',5'-phosphodiester bond formation and also as activated precursor to desired mono-, di-, or triphosphate.
Imai and Torrence, 1981b	The completely protected product of a triester synthesis was converted to free 5'-OH by MMT removal and then phosphorylated with bis(2,2,2-trichloroethyl)phosphorochloridate, followed by I ₂ oxidation to yield the 5'-monophosphate, which was then converted to the 5'-triphosphate through the phosphoroimidazolidate reaction with bis(tri- <i>n</i> -butylammonium)diphosphate.	

MMT, *p*-monomethoxytrityl.

treatment with 1000 U/mL of rIFN- α A, the isoform p40 was induced to twice the extent of p100. In contrast, rIFN- γ at 5000 U/mL induced both isoforms equally (Rosenblum *et al.*, 1988). The induction of the p69 and p100 isoforms has also been studied in Daudi, fibroblast, and colon adenocarcinoma cells *in vitro* and *in vivo* (Witt *et al.*, 1993a,b). There were significant differences in induction between cell types with IFN- α_2 , IFN- β_{ser} , and IFN- γ . In addition, differential induction occurred in PBMC lysates taken from normal donors cultured with IFNs *in vitro* and in cancer patients being treated with IFN- α_2 or IFN- β_{ser} . Isoforms also are present in other species (Shimizu *et al.*, 1984).

2-5A synthetase levels do not always correlate with the extent of the antiviral state (Lewis, 1988). The inhibition of encephalomyocarditis virus (EMCV) replication seems to correlate with 2-5A synthetase induction (Baglioni *et al.*, 1979). Likewise, mengovirus and vaccinia replication inhibitions were characterized by high 2-5A synthetase levels (Chebath *et al.*, 1987b; Grun *et al.*, 1987). Resistance to ve-

sicular stomatitis virus (VSV) replication, however, can occur in the absence of a detectable increase in 2-5A synthetase activity (Hovanessian *et al.*, 1981b; Meurs *et al.*, 1981). Inhibition of influenza virus, herpes simplex virus, or herpes delta virus replication does not seem to be a result of 2-5A synthetase activity (De Benedetti *et al.*, 1987; Taylor *et al.*, 1995; McNair *et al.*, 1994). Suppression of 2-5A synthetase induction by persistent infection with mumps virus has also been documented (Fujii *et al.*, 1990).

When IFNs that confer antiviral activity on heterologous cells were examined, 2-5A synthetase levels also were found to be elevated. Thus, chicken IFN conferred resistance to quail QT35 cells, while raising their 2-5A synthetase levels (Fulton *et al.*, 1995). Human IFN- α A showed both antiviral and 2-5A synthetase inducing activity in MDBK and WISH cells, but neither activity in L929 cells (Chelbi-Alix and Thang, 1987).

Enhanced induction of 2-5A synthetase may play a part in the well-described potentiation of IFN action by febrile

TABLE 4. Synthetic Analogues of 2-5A

Analogue	Reference
Modifications to the glycone moieties:	
3'-O-Methylated	
(pp)p5' A2' p5' A2' p5' A	den Hartog <i>et al.</i> , 1981
(pp)p5' Am2' p5' Am2' p5' A	
Uronic acid analogue	Kinjo <i>et al.</i> , 1992
Xylofuranosyl analogue	
(xylo-A)2' p5' (xylo-A)2' p5' (xylo-A)	Gosselin and Imbach, 1981, 1982
β -Alanyltyrosine conjugate	
ppp5' A2' p5' A2' p5' A2' p5' A- β -Ala-Tyr [2'-terminal ribose oxidized with IO ₄ ⁻ to provide dialdehyde that was coupled with β -alanine-tyrosine dipeptide]	Sawai, 1985
2'-Terminus modifications	
2-5A-antisense	Torrence <i>et al.</i> , 1993; Lesiak <i>et al.</i> , 1993
A2' p5' A2' p5' X	Engels, 1980
X = 3'dA, 2'dA, 2',3'ddA, araA	
(pp)p5' A2' p5' A2' p5' A2' p5' A*-n-hexyl [2'-terminal ribose oxidized with IO ₄ ⁻ to provide dialdehyde that was coupled with 1-aminohexane]	Imai <i>et al.</i> , 1982a
A2' p5' A2' p5' [2' (3')-O-palmitoyladenine]	Pressova and Smrt, 1989
A2' p5' A2' p5' [2' (3')-O-(1-methoxyhexadecylidene)adenosine]	
p5' A2' p5' A2' p5' A2' (o-phosphoglyceryl)	Bayard <i>et al.</i> , 1984
p5' A2' p5' A2' p5' [2' (3')-O-(1-methoxyhexadecylidene)adenosine]	
2-5 "core" trimer analogues with modified 2'-terminal nucleosides including 9-(2',3'-anhydro- β -D-ribofuranosyl)-adenine, 9-(3'-deoxy- β -D-glycero-pent-3-enofuranosyl)-adenine, 9-(3'-halo-3'-deoxy- β -D-xylofuranosyl)adenine, and 9-(3'-azido-3'-deoxy-D-xylofuranosyl)adenosine	Herdewijn <i>et al.</i> , 1991
A2' p5' A2' p5' [2',3'-di-O-(2-carboxyethyl)ethylidene]adenosine tyrosine methyl ester	Kvasyuk <i>et al.</i> , 1996
Acyclic analogue	
HOCH ₂ CH(OH)OCH(Ade)CHCH ₂ OP(O)(OH)- as repeating unit in trinucleotide	Mikhailov and Pfeleiderer, 1985
5'-Amino analogues	
5'-amino-5'-deoxyadenylyl(2'-5')adenylyl(2'-5')adenosine	Kvasyuk <i>et al.</i> , 1995
5'-deoxy-5'-(hexadecanoylamino)adenylyl(2'-5')adenosine	
5'-Amino and 2'-terminal acyclic modifications	
5'-amino-5'-deoxyadenylyl(2'-5')adenylyl(2'-2'')-N ⁹ -[(2''-hydroxyethoxy)methyl]adenine	
5'-deoxy-5'-(hexadecanoylamino)adenylyl(2'-5')-adenylyl(2'-2'')-N ⁹ -[(2''-hydroxyethoxy)methyl]adenine	
2-5A "Core" analogues substituted with TBDMS groups	
A2' p5' A2' p5' A3' (TBDMS)	Schirmeister and Pfeleiderer, 1994
A[3'-TBDMS]2' p5' A2' p5' A3' [TBDMS]	
3' dA2' d5' A2' d5' A3' [TBDMS]	
3'-Deoxyadenosine (cordycepin) analogues	
(3'dA)2' p5' (3'dA)2' p5' (3'dA)	Wasner <i>et al.</i> , 1996
Adducts consisting of covalent conjugation at the 5' and 2' termini of the above 3'-dA analogue of 2-5A with vitamins A, D ₂ , E, and the lipids 1,2-di-O-palmitoylglycerol and 1,2-di-O-hexadecylglycerol	
3'-deoxyadenylyl-(2'-5')-3'-deoxyadenylyl-(2'-5')adenosine	Charubala and Pfeleiderer, 1980b
(2'-5')-3'-O-(2-hydroxyethyl)adenosine and 3'-deoxyadenylyl-(2'-5')-3'-deoxyadenylyl-(2'-5')-3'-O-[2-[(cholest-5-en-3 β -yloxy)-carbonyloxy]ethyl]adenosine	Horndler and Pfeleiderer, 1996
3'-deoxyadenylyl-(2'-5')-3'-deoxyadenylyl-(2'-5')-2'-O-[[2-(cholest-5-en-3 β -yloxy)carbonyl]-ethyl]-carbonyl]-3'-deoxyadenosine and 5'-O-[[2-(cholest-5-en-3 β -yloxy)carbonyl]ethyl]-carbonyl]-3'-deoxyadenylyl-(2'-5')-3'-deoxyadenylyl-(2'-5')deoxyadenosine	Wasner <i>et al.</i> , 1994
(pp)p(3'dA)2' p5' (3'dA)2' p5' (3'dA)2' p5' (3'dA)	Sawai <i>et al.</i> , 1983
(3'dA)2' p5' (3'dA)2' p5' (3'dA)	Charubala and Pfeleiderer, 1980b; Nyilas <i>et al.</i> , 1986
3'-deoxyadenylyl-(2'-5')-3'-deoxyadenylyl-(2'- ω)-9-(ω -hydroxyalkyl)adenines with variation in number of methylenes in hydroxyalkyladenine chain	Mikhailov <i>et al.</i> , 1991
Sequence-specific 3'-deoxyadenosine analogues	Torrence <i>et al.</i> , 1988
(pp)p5' (3'dA)2' p5' A2' p5' A	
(pp)p5' A2' p5' (3'dA)2' p5' A	
(pp)p5' A2' p5' A2' p5' (3'dA)	
p5'(3'dA)2-p5'A2'p5'(3'dA)	Charubala <i>et al.</i> , 1989a

(continued)

TABLE 4. Continued

Analogue	Reference
γ -Phosphorothioate and β,γ -difluoromethylene-phosphonate analogues of 2-5A tetramer; also poly(L-lysine) conjugate of γ -phosphorothioate 2-5A analogue (all obtained by enzymatic synthesis)	Bisbal <i>et al.</i> , 1987
ppp5'A2'p5'A2'p5'A2'p5'A-poly(L-lysine) [coupling vis periodate oxidation of 2-5A followed by Schiff base formation and cyanoborohydride reduction]	Bayard <i>et al.</i> , 1986
5'-Capped 2-5A analogues including A5'ppp5'A2'p5'A2'p5'A, A5'ppp5'A2'p5'A2'p5'A A2'pppp5'A2'(p5'A2') _n with n = 1-3	Imai and Torrence, 1984
ppp5'A2'p5'A2'p5'N, with N = rC, rG, rU, dT, dC, dG, dA (enzymatic synthesis)	Drocourt <i>et al.</i> , 1982
ppp5'A2'(p5'A2') ₂ p5'Aox-red	Bayard <i>et al.</i> , 1985
ppp5'A2'(p5'A2') ₃ p5'A2'pOCH ₂ -CH(OH)CH ₂ OH	
(pp)p(3'dA)2'p5'(3'dA)2'p5'(3'dA)	Sawai <i>et al.</i> , 1983; Nyilas <i>et al.</i> , 1986
p5'(3'dA)2'p5'A2'p5'(3'dA)	
2'-Fluoro-2'-deoxyadenosine (A _F) analogues (pp)5'A _F 2'p5'A _F 2'p5'A _F (pp)5'A _F 2'p5'A2'p5'A (pp)5'A2'p5'A _F 2'p5'A (pp)5'A2'p5'A2'p5'A _F (pp)5'A2'p5'AF2'p5'A _F (pp)5'A _F 2'p5'A2'p5'A _F	Kovacs <i>et al.</i> , 1993, 1995
Arabinosyladenosine analogues (ara-A)2'p5'(ara-A)2'p5'(ara-A) (ara-A)2'p5'A2'p5'(ara-A) A2'p5'(ara-A)2'p5'(ara-A) (ara-A)2'p5'A2'p5'A A2'p5'A2'p5'(ara-A) (ara-A)2'p5'(ara-A)2'p5'A	Kwiatkowski <i>et al.</i> , 1981 Kwiatkowski <i>et al.</i> , 1982
Aristeromycin analogue	Sawai <i>et al.</i> , 1985b
2-Terminus with 3'-amino-3'dA or 3'-tetradecanamido-3'-dA A2'p5'A2'p5'(3'dANH ₂) A2'p5'A2'p5'(3'dANHCO-(CH ₂) ₁₂ CH ₃)	Pfleiderer <i>et al.</i> , 1994 Visser <i>et al.</i> , 1986
Modifications to the backbone and 5'-mono-/triphosphate moiety:	
Mannose phosphate adduct Human-pp5'A2'p5'A2'p5'A	Lesiak <i>et al.</i> , 1989
β,γ -Methylenephosphonate pCH ₂ ppA2'p5'A2'p5'A	den Hartog <i>et al.</i> , 1981
Linkage isomers A2'p5'A3'p5'A A3'p5'A2'p5'A (pp)p5'A3'p5'A3'p5'A (pp)p5'A2'p5'A3'p5'A (pp)p5'A3'p5'A2'p5'A	Charubala and Pfeleiderer, 1980a Lesiak <i>et al.</i> , 1983
P ³ -n-decylaminotriphosphate analogue n-decyl-NH-ppp5'A2'p5'A2'p5'A	Kariko and Ludwig, 1985
Methylthiophosphate analogue H ₃ CSp5'A2'p5'A2'p5'A2'ppOCH ₃	Watling <i>et al.</i> , 1985
Phosphorodithioate analogue p5'A2'(s2p)5'A2'(s2p)5'A	Beigelman <i>et al.</i> , 1995
Phosphorothioate analogues (Rp)-P-thioadenylyl(2'-5')adenosine (Sp)-P-thioadenylyl(2'-5')adenosine (Rp)-P-thioadenylyl(2'-5')-(Rp)-P-thioadenylyl(2'-5')adenosine [±] (Rp)-P-thioadenylyl(2'-5')-(Sp)-P-thioadenylyl(2'-5')adenosine [±] (Sp)-P-thioadenylyl(2'-5')-(Rp)-P-thioadenylyl(2'-5')adenosine [±] (Sp)-P-thioadenylyl(2'-5')-(Sp)-P-thioadenylyl(2'-5')adenosine [±] also diastereomeric tetramers Adenylyl(2'-5')-(Rp)-P-thioadenylyl(2'-5')adenosine Adenylyl(2'-5')-(Sp)-P-thioadenylyl(2'-5')adenosine (Rp)-P-thio-3'-deoxyadenylyl(2'-5')-(Rp)-P-thio-3'-deoxyadenylyl(2'-5')-3'-deoxyadenosine	Charubala and Pfeleiderer, 1992 Charubala and Pfeleiderer, 1988 Charubala and Pfeleiderer, 1992 also Battistini <i>et al.</i> , 1992 also Shimazu <i>et al.</i> , 1992 also Nelson <i>et al.</i> , 1984; Charubala <i>et al.</i> , 1991 De Vroom <i>et al.</i> , 1987 Sobol <i>et al.</i> , 1993a

(continued)

TABLE 4. Continued

Analogue	Reference
(Rp)-P-thio-3'-deoxyadenylyl(2'-5')-(Sp)-P-thio-3'-deoxyadenylyl(2'-5')-3'-deoxyadenosine	
(Sp)-P-thio-3'-deoxyadenylyl(2'-5')-(Rp)-P-thio-3'-deoxyadenylyl(2'-5')-3'-deoxyadenosine	
(Sp)-P-thio-3'-deoxyadenylyl(2'-5')-(Sp)-P-thio-3'-deoxyadenylyl(2'-5')-3'-deoxyadenosine	
Modification to the aglycone moiety:	
Inosine analogues	
12'p5'12'p5'1 and tetramer	Charubala and Pfeleiderer, 1982
(pp)p5'12'p5'12'p5'1	Torrence <i>et al.</i> , 1984
(pp)p5'A2'p5'A2'p5'1	Imai and Torrence, 1985
(pp)p5'A2'p5'12'p5'A	Imai <i>et al.</i> , 1985
(pp)p5'12'p5'A2'p5'A	
Uridine analogues	
(pp)p5'U2'p5'U2'p5'U	Torrence <i>et al.</i> , 1984
(pp)p5'A2'p5'A2'p5'U	Kitade <i>et al.</i> , 1991a
(pp)p5'A2'p5'U2'p5'A	
(pp)p5'U2'p5'A2'p5'A	
Cytidine analogues	
(pp)p5'C2'p5'C2'p5'C	Torrence <i>et al.</i> , 1984
p5'A2'p5'C2'p5'A	K. Lesiak and P. F. Torrence, unpublished observations
7-Deazaadenosine (tubercidin) analogues	
(pp)p5'(c7A)2'p5'(c7A)2'p5'(c7A)	Jamouille <i>et al.</i> , 1984, 1987
(pp)p5'A2'p5'A2'p5'(c7A)	
(pp)p5'A2'p5'(c7A)2'p5'A	
(pp)p5'(c7A)2'p5'A2'p5'A	
(pp)p5'A2'p5'(c7A)2'p5'(c7A)	
Guanosine analogue	
(pp)pG2'p5'(c7A)2'p5'(c7A)	Jamouille and Torrence, 1986
A2'p5'A2'p5'G	Kvasyuk <i>et al.</i> , 1987
A2'p5'G2'p5'A	
8-Bromoadenosine analogues	
(pp)p5'(br8A)2'p5'(br8A)2'p5'(br8A) and longer oligos	Lesiak and Torrence, 1986
(pp)p5'A2'p5'A2'p5'(br8A)	Yoshida and Takaku, 1986a,b
(pp)p5'A2'p5'(br8A)2'p5'A	Lesiak and Torrence, 1987
(pp)p5'(br8A)2'p5'A2'p5'A	
(pp)p5'A2'p5'(br8A)2'p5'(br8A)	
(pp)p5'A2'p5'A2'p5'(br8A)2'p5'(br8A)	Torrence <i>et al.</i> , 1992
8-Methyladenosine analogues	
(pp)p5'(m8A)2'p5'(m8A)2'p5'(m8A)	Kitade <i>et al.</i> , 1991b
(pp)p5'(m8A)2'p5'A2'p5'A	
(pp)pA2'p5'(m8A)2'p5'A	
(pp)pA2'p5'A2'p5'(m8A)	
8-Hydroxyadenosine analogues	
A2'p5'A2'p5'(ho ⁸ A)	Yoshida and Takaku, 1986b
5'-Substituted pyrimidine nucleotide analogues	
A2'p5'A2'p5'R	Herdewijn <i>et al.</i> , 1987a, 1989a
R = 5-bromovinyl-2'-deoxyuridine, 5-fluoro-2'-deoxyuridine, acyclovir	
3'-Azido- and 3'-amino-substituted xylofuranosyl analogues	
2-5A "core" trimer analogues containing 9-(3-azido-3-deoxy-β-D-xylofuranosyl)adenine or 9-(3-amino-3-deoxy-β-D-xylofuranosyl)adenine	Herdewijn <i>et al.</i> , 1987b, 1989b
2-Azidoadenosine analogue	Suhadolnik <i>et al.</i> , 1988
ppp5'(N ₃ ² A)2'p5'(N ₃ ² A)2'p5'(N ₃ ² A)	
8-Azidoadenosine analogues	Suhadolnik <i>et al.</i> , 1988
ppp5'(N ₃ ⁸ A)2'p5'(N ₃ ⁸ A)2'p5'(N ₃ ⁸ A)	Charubala <i>et al.</i> , 1989b
A2'p5'A2'p5'(N ₃ ⁸ A)	
εA, ethenoadenosine (3-β-D-ribofuranosylimidazo[2.1-i]purine) analogue	Lesiak and Torrence, 1983
(pp)p5'εA2'p5'εA2'p5'εA	

TBDMS, t-butyl-dimethylsilyl.

temperatures. Hyperthermia (45°C) induced 2-5A synthetase in MDBK cells and also released a heat shock-induced factor (HSIF), which, in turn, could induce 2-5A synthetase in human WISH cells (Chousterman *et al.*, 1987). This HSIF has since been shown to be an atypical bovine IFN (Gachet *et al.*, 1993). The IFN-mediated antiviral effect was enhanced 3- to 10-fold in Type I IFN-treated HL-60 and in WISH cells cultured at 39.5°C, and this was associated with a 2- to 3-fold increase in the p40 2-5A synthetase (Chang and Wu, 1991). Stability assays showed that the mRNA half-life was selectively extended from 2 to 4 hr, although the half-lives of poly(A)⁺ RNA and β -actin mRNA remained unchanged.

Glucocorticoids, at concentrations capable of inhibiting DNA synthesis and cell division, induced 2-5A synthetase in Daudi and Raji cells (Krishnan and Baglioni, 1980b). Cortisol inhibited glucocorticoid induction, but did not inhibit 2-5A synthetase induction by IFN. 2-5A synthetase activity also was increased in the chick oviduct after estrogen withdrawal (Stark *et al.*, 1979). The antiestrogen tamoxifen, at levels that were growth inhibitory, increased 2-5A synthetase and RNase L levels in a cell line that was estrogen receptor positive (CG5), but not in one that was receptor negative (EVSA-T) (Viano *et al.*, 1989).

A number of autocrine growth factors can induce 2-5A synthetase alone or in combination with various IFNs. Additive 2-5A synthetase-inducing effects of IFN- γ and transforming growth factor (TGF)- α have been observed in A431 cells (Wietzerbin *et al.*, 1990), although the question of whether or not TGF- α could induce 2-5A synthetase in the absence of IFN was not addressed (Kumar and Mendelsohn, 1990). Epidermal growth factor-urogastrone caused a 3- to 5-fold induction of 2-5A synthetase in human fibroblasts, which was not reversed with anti-IFN- β antibodies (Lin *et al.*, 1983). Platelet-derived growth factor (PDGF) induced 2-5A synthetase in murine BALB/c 3T3 cells, but not in the presence of mono- or polyclonal antibodies to murine IFN- β (Garcia-Blanco *et al.*, 1989). Tumor necrosis factor- α , in combination with either IFN- β or IFN- γ , exhibited significant synergism with regard to 2-5A synthetase induction (Mestan *et al.*, 1988; Wietzerbin *et al.*, 1990). Nerve growth factor (NGF) induced 2-5A synthetase from 5- to 25-fold in the rat pheochromocytoma cell line (PC12), but the basis for the effect was unclear (Saarma *et al.*, 1986).

Ever since the finding that treatment of Daudi cells with IFN- α or IFN- β caused transient increases in the level of diacylglycerols (Yap *et al.*, 1986), there has been significant interest in the contribution of protein kinase C (PKC) to the induction of 2-5A synthetase. Some investigators have found that (1) PKC activators such as 12-O-tetradecanoylphorbol-13-acetate (TPA) can potentiate the action of IFN in HL-60, Daudi and HeLa cells; (2) PKC inhibitors such as staurosporine or H7 can inhibit 2-5A synthetase induction; (3) down-regulation of PKC by TPA can decrease 2-5A synthetase induction (Faltynek *et al.*, 1989; Raber *et al.*, 1991; Chang *et al.*, 1992; Percario *et al.*, 1995). To the con-

trary, others have observed decreases in 2-5A synthetase activity after short-term treatment with TPA (Yan *et al.*, 1989). Additional research needs to be done to clarify the importance of PKC in the induction of 2-5A synthetase by IFN.

Vasoactive intestinal peptide has been shown to induce 2-5A synthetase in glial cells and HT-29 cells, but not in neurons (Chelbi-Alix *et al.*, 1994). The induction of 2-5A synthetase was preceded by the synthesis of heat shock protein (hsp) 70 and was abolished by treatment by anti-IFN- α/β . Cyclic AMP also induced 2-5A synthetase mRNA on a transcriptional level (Itkes *et al.*, 1984b). Although there are no sites in the 2-5A synthetase 5'-flanking region analogous to the cAMP-responsive element TGACGTCA, several other potential transcription binding factor sites do exist (Itkes, 1994). Interleukin (IL)-6 has also been found to exhibit synergism with Type I IFN in M1 cells, possibly by inducing DNA binding factors (Cohen *et al.*, 1991). IL-6 in M1 cells induced different 2-5A synthetase isozymes than IFN, including one that is cell-surface associated. Murine IFN- α/β antibodies do not completely abolish 2-5A synthetase induction mediated by IL-6 (Gothelf *et al.*, 1991).

A number of drugs and chemicals have been reported to increase 2-5A synthetase activity (Table 6), but whether or not the intermediate production of IFN was responsible for the induction usually was not addressed. Other agents have been found to decrease 2-5A synthetase induction. These include gangliosides (Krishnamurti *et al.*, 1982), arachidonic acid analogues (Menon *et al.*, 1990), glucose (Onishi *et al.*, 1986) and concanavalin A (Faltynek *et al.*, 1988).

Since the level of 2-5A synthetase mRNA decreased during apoptosis, the 2-5A synthetase protein was surmised not to play a direct role in cell death through the production of 2-5A (Kelve *et al.*, 1994). However, more recent experiments suggest a role for the 2-5A system in apoptosis (Castelli *et al.*, 1997; see Section 6.25).

3.3. Role of 2-5A Synthetase in the Antiproliferative Effects of Interferon

There is considerable evidence documenting negative growth regulatory effects of IFNs. Whether or not the 2-5A system plays a direct role through production of 2-5A, followed by RNase L-mediated cleavage of cellular mRNAs, is somewhat less clear. 2-5A synthetase activity has been found to be low in normal proliferating diploid Syrian hamster (FC13) cells, 3-fold higher in density-arrested FC13 cells, and uniformly low in their neoplastically transformed derivative BP6T cells in the absence of autocrine IFN production (Hassel and Ts'0, 1992). Similar results have been obtained in the BSC1 line, in which the 2-5A synthetase levels were greatest in quiescent cells (Kimchi *et al.*, 1981). This was also the case in Namalva cells, in which 2-5A synthetase levels were highest during serum-starvation conditions (Krishnan and Baglioni, 1981), and also in JLS-V9R cells, in which 2-5A synthetase levels were highest at

TABLE 5. 2-5A Synthase Identified in Various Species by Tissue or Cell Type

Species	Tissue or cell type	Reference
Chick	Embryo	West and Ball, 1982
	Embryo	Sekellick <i>et al.</i> , 1990
	Embryo fibroblasts	Lab <i>et al.</i> , 1982
	Embryo tendon fibroblasts	Oikarinen, 1982
	Oviduct	Stark <i>et al.</i> , 1979
	Thyroid epithelium	Kuhr <i>et al.</i> , 1994
	Myoblasts	Birnbaum <i>et al.</i> , 1990
	Erythrocytes	Sokawa and Sokawa, 1986
Quail	QT35	Fulton <i>et al.</i> , 1995
Duck	Serum	Akagi <i>et al.</i> , 1992
Cow	Endometrium (stroma, epithelium)	Schmitt <i>et al.</i> , 1993
	MDBK	Short <i>et al.</i> , 1992
Pig	Spleen homogenate	Shimizu and Sokawa, 1983
	Trophoblast (none detected)	D'Andrea <i>et al.</i> , 1994
	Endometrium	Short <i>et al.</i> , 1992
	Enterocytes, testicular cells	Bosworth <i>et al.</i> , 1989
Dog	Liver homogenates	Etienne-Smekens <i>et al.</i> , 1981
Monkey	CV-1	Rice <i>et al.</i> , 1985
	Kidney	Bannai <i>et al.</i> , 1985
	Kidney, PBL	Bannai, 1986
Mouse	Various tissues	Krishnan and Baglioni, 1980a
	Splenic lymphocytes	Shimizu and Sokawa, 1979
Guinea pig	Macrophages	Wang and Wu, 1986
Hamster	FC13	Hassel and Ts'O, 1992
Rabbit	Reticulocytes	Suhadolnik <i>et al.</i> , 1990
	Reticulocytes	Li <i>et al.</i> , 1990
Reptiles	Reticulocytes	Kariko <i>et al.</i> , 1987a
	Liver, kidney, heart homogenates	Cayley <i>et al.</i> , 1982b
Viruses	VSV, M-MuLV	Wallach and Revel, 1980

confluency (Jacobsen *et al.*, 1983b). Stable transfection of a human 2-5A synthetase cDNA into T98G cells was associated not only with EMCV resistance, but also with a reduced rate of cellular proliferation (Rysiecki *et al.*, 1989). Levels of 2-5A synthetase during the cell cycle have been examined (Wells and Mallucci, 1985). Using mouse embryo tertiary fibroblasts progressing synchronously through the cell cycle as determined by fluorescence-activated cell sorting, 2-5A synthetase levels were found to be low in quiescent cultures, as well as during G1 and early S. They then rose 10-fold in late S and decreased sharply in G2. A similar, though quantitatively lower pattern, was found for RNase L.

Induction of 2-5A synthetase preceded the antiproliferative effects of IFN in Moloney murine sarcoma virus (MSV)-transformed NIH 3T3 cells (Salzberg *et al.*, 1990b). Treatment of A431 cells with IFN- γ resulted in growth inhibition that was kinetically correlated to significant increases

TABLE 6. Drugs or Chemicals that Act to Increase 2-5A Synthetase Activity

Drug	Tissue or cell type	Reference
Phenobarbital	Rat liver nuclei	Liu and Owens, 1987
Pentostatin	Serum and PBMCs after <i>in vivo</i> administration	Ho <i>et al.</i> , 1992
CL246,738	PBMCs after <i>in vivo</i> administration	Litton <i>et al.</i> , 1990
Tyrosine kinase inhibitors	Melanoma cells	Ralph <i>et al.</i> , 1995
Retinoic acid	NB-4 cells	Kumar and Korutla, 1995
	WISH, Namalva	Bourgeade and Besacon, 1984
Indomethacin	Serum after <i>in vivo</i> administration	Andreone <i>et al.</i> , 1994
Bile acids	Human PBMCs	Podevin <i>et al.</i> , 1995
Imiquimod	PBMCs after <i>in vivo</i> administration	Witt <i>et al.</i> , 1993b
Ethanol	MDBK cells	Chelbi-Alix and Chousterman, 1992
Dimethyl sulfoxide	HL-60 cells	Schwartz and Nilson, 1989
Theophylline	3T3 cells	Itkes <i>et al.</i> , 1984a,b

in 2-5A synthetase isozymes (Kumar and Mendelsohn, 1989). 2-5A synthetase p40 and p100 isoenzymes were induced up to day 4, while the p67 isozyme induced late and remained high until day 7 after treatment. IFN- γ has been shown to potentiate the response of T-cells to mitogens, but not to induce an antiviral state (Landolfo *et al.*, 1988). However, in this same study, IFN- α/β , but not IFN- γ , induced increases in 2-5A synthetase. Human IFN- $\alpha 2$ has been shown to cause blast cyto-reduction in peripheral blood and marrow of juvenile acute myelogenous leukemia patients in concert with an increase in 2-5A synthetase levels (Freedman *et al.*, 1986). Salzberg *et al.* (1990b) found that IFN treatment of MSV-transformed NIH 3T3 fibroblasts resulted in decreased rates of cell growth, protein synthesis, and cloning efficiency, with a parallel 10-fold rise in 2-5A synthetase levels. Recovery occurred only when the level of IFN treatment was significantly decreased and 2-5A synthetase levels dropped also.

Antiviral and growth inhibitory effects seem to be dissociated at the genetic level. A mutant murine cell line (Itk⁻aprt⁻), which was unable to mount an antiviral response with IFN- β treatment, remained growth-inhibited by IFN- β (Shan and Lewis, 1989). A human cell type has been described that expressed both IFNABR receptor subunits Jak 1 and Tyk 2, had adequate levels of Stat 1, Stat 2, and Stat 3, and could mount an antiviral response, but remained insensitive to the growth-inhibitory effects of IFN. On this basis, a human chromosome 21-encoded component, which may confer IFN-inducible growth inhibition to cells in the context of a high-affinity interaction through IFNABR- α/β , has been postulated (Ghislain *et al.*, 1995).

Suppression of *c-myc* may play a role in IFN's growth inhibitory properties. Autocrine production of IFN resulted in *c-myc* suppression and the G₀/G₁ arrest that occurred during the terminal differentiation of hematopoietic cells (Resnitzky *et al.*, 1986). During growth arrest and differentiation, down-regulation of *c-myc* and 2-5A synthetase induction occurred in M1 cells, but not in murine radiation-induced acute myelogenous leukemia cells (Resnitzky *et al.*, 1992). Human IFN- β ser17 inhibited growth of human colon-carcinoma DLD-1 clone A cells *in vitro* and expression of the *c-myc* proto-oncogene through post-transcriptional destabilization of the *c-myc* mRNA (Chatterjee and Savarase, 1992).

Other investigators have not found a correlation between growth inhibition and 2-5A system activity. Induction of 2-5A synthetase in human immunodeficiency virus (HIV)-infected T-cells was not correlated with growth inhibition, but was correlated with the antiviral state (Fujii *et al.*, 1992). Although IFN- β resulted in a large increase in human prostatic JCA-1 cell 2-5A synthetase activity, neither increased p68 dsRNA-dependent protein kinase (PKR) levels nor RNase L activation could be demonstrated (Nakajima *et al.*, 1994). In IL-1 mitogen-stimulated smooth muscle cell cultures, IFN- γ reduced the rate of [³H]thymidine incorporation and delayed the time to peak level of *c-fos* RNA; however, the kinetic characteristics of 2-5A synthetase induction in these same cultures made it unlikely that 2-5A synthetase accounted for the cytostatic effects of IFN- γ (Warner *et al.*, 1989). In alveolar pulmonary tumor cells (A459) maintained in organotypic culture, only IFN- α 2 and IFN- γ resulted in an antiproliferative effect, but IFN- α , IFN- β , and IFN- γ all caused an increase in 2-5A synthetase activity (Martyre *et al.*, 1988).

3.4. 2-5A Synthetase and Differentiation

2-5A synthetase has been found to be positively correlated with differentiation and the maturational process. Induction of differentiation with chemicals or cytokines frequently has been associated with 2-5A synthetase induction. For instance, 2-5A synthetase activity was found to reach a peak 26 hr after hexamethylene bis-acetamide induction of terminal differentiation of Friend erythroleukemia cells, even in the presence of anti-IFN- α/β (Salzberg *et al.*, 1996). A 27-fold increase in 2-5A synthetase has been observed in human HL-60 cells induced to differentiate with dimethyl sulfoxide, and this increase was ablated by anti-IFN- α (Schwartz and Nilson, 1989). Induction of differentiation in U937 cells by hydroxyvitamin D₃ was associated with autocrine IFN production and increased in 2-5A synthetase (Testa *et al.*, 1988). IL-6 induced differentiation of murine M1 cells into mature macrophages coincident with induction of IFN- β (Bickel *et al.*, 1990). However, the observed induction of 2-5A synthetase by IL-6 occurred in the presence of anti-IFN- β antibodies and cycloheximide, and was characterized by the presence of two mRNA species (1.7 and 2.4 kb), whereas IFN- β induced only the 1.7 kb

mRNA species. This may have resulted from a direct induction of binding factors that act on the IFN-stimulatable response element (ISRE) (Cohen *et al.*, 1991).

2-5A synthetase activity increases have been found in a wide variety of cell types during differentiation. Differentiation of skeletal muscle cells in several species has been found to be associated with 2-5A synthetase induction and 2-5A pppA₃ formation and with a decrease in *c-myc* expression (Birnbaum *et al.*, 1990). IFN- β 2 induced growth arrest and differentiation of M1 and U937 cells, as well as 2-5A synthetase induction that was synergized by IFN- γ (Chen *et al.*, 1988). M1 variants that were resistant to IL-6-induced differentiation could not activate the 2-5A synthetase gene. In mouse condylar cartilage, 2-5A synthetase levels have been found to be greatest in proliferating, undifferentiated chondrocytes, whereas fully differentiated chondrocytes showed lower levels (Maor *et al.*, 1990). In primary chronic lymphocytic leukemia cells, there was a close correlation between 2-5A synthetase induction and induction of blast transformation (Ostlund *et al.*, 1986). In clones not exhibiting IFN-induced 2-5A synthetase increases, blast transformation did not occur. 2-5A synthetase levels and 10 differentiation markers were monitored in the human monocyte cell line U937 and the promyelocytic cell line HL-60 during cellular maturation (Ferus *et al.*, 1985). 2-5A synthetase levels increased prior to many of the differentiation markers and rose during the maturational process in both cell lines.

Some investigators have not found a causal connection between 2-5A synthetase induction and differentiation. The human neuroblastoma cell line LAN-5 was sensitive to the differentiation-promoting effects of IFN- γ (Corrias *et al.*, 1995). All isoforms of 2-5A synthetase were induced in this cell line by IFN- γ , including clones that were resistant to the neuronal maturation effects. Sodium butyrate, an inducer of differentiation in Burkitt's lymphoma cells, was found to cause a rapid decrease in *c-myc* mRNA, as well as an increase in 2-5A synthetase mRNA; however, the time course of 2-5A synthetase induction precluded a direct effect (Polack *et al.*, 1987).

3.5. Assay of 2-5A Synthetase

A variety of methods have been advanced towards assaying 2-5A synthetase, and these assays involve three separate stages: (1) isolation of 2-5A synthetase from cells, (2) the synthesis of 2-5A oligonucleotide, and (3) measurement of the 2-5A oligonucleotide concentration (Table 7).

The enzymatic synthesis of 2-5A may be performed in the solution phase; however, high 2-5A degrading activities in certain cell extracts, e.g., HeLa, make this problematic. Adsorption of lysate-derived 2-5A synthetase to an affinity support followed by ATP, Mg²⁺, K⁺, and sometimes dsRNA supplementation with subsequent 2-5A synthesis, has been most common (Hovanessian *et al.*, 1981a). Poly(I):poly(C) linked to agarose, sepharose, cellulose, or paper has been the most commonly used affinity matrix. 2-5A

TABLE 7. Methods Used for Assaying 2-5A Produced by 2-5A Synthetase

1. Biological activity
 - A. Inhibition of protein synthesis. Evaluation of the concentration required to effect a 50% reduction in an mRNA-programmed cell-free system (Johnston *et al.*, 1980a; Williams *et al.*, 1981).
 - B. Nuclease activation assay. Detection of 2-5A is achieved through RNase L activation. RNA cleavage is observed by:
 - (i) Gel electrophoresis. Specific (Wreschner *et al.*, 1981a) or general (Williams *et al.*, 1981) RNA cleavages may be observed.
 - (ii) Acid precipitable counts (Baglioni *et al.*, 1981a).
 - (iii) Degradation of radiolabelled poly(U) by RNase L:core (2-5A) cellulose in the presence of 2-5A (Silverman, 1985).
2. 2-5A produced using radiolabelled ATP and separated from excess ATP using one of the following techniques
 - A. HPLC (Brown *et al.*, 1981; Knight *et al.*, 1980) utilizes reverse-phase C₁₈ columns and phosphate buffers at pH 7 to examine enzymatically synthesized 2-5A mixtures.
 - B. Column chromatography
 - (i) Ion exchange media such as DEAE-sephadex or -cellulose enable ATP to be separated from more highly charged 2-5As or their cores (Minks *et al.*, 1979a; Hovanessian *et al.*, 1981a; Wells *et al.*, 1984; Miller and Bell, 1995).
 - (ii) Alumina columns bind [³²P] strongly, but dimer to tetramer 2-5A is recovered almost quantitatively, enabling the detection of small quantities of 2-5A synthetase (Merlin *et al.*, 1981; Revel *et al.*, 1981; Schattner *et al.*, 1981).
 - (iii) SepPak C₁₈ cartridges may be used to purify (2',5')oligo(A) from excess ATP (Hassel and Ts'O, 1994).
 - C. TLC
 - (i) Untreated 2-5A separated using PEI-cellulose (Justesen *et al.*, 1980).
 - (ii) Bacterial alkaline phosphatase digests heterogeneous 2-5A mono-, di-, and triphosphates to form the corresponding cores that are then separated with PEI-cellulose (Verhaegen *et al.*, 1980; Minks *et al.*, 1979a).
 - (iii) Hexokinase/glucose treatment to form 2-5A diphosphates (Dougherty *et al.*, 1980).
 - D. DEAE-paper binding (Ball and White, 1979a).
 - E. Electrophoresis
 - (i) High-voltage paper electrophoresis (Kerr *et al.*, 1977).
 - (ii) Denaturing 20% polyacrylamide gel electrophoresis separates 2-5A, enabling a large number of samples to be rapidly processed in parallel (Miele *et al.*, 1991).
 - (iii) Capillary isotachophoresis has been utilized to measure ATP at the beginning and end of a reaction on poly(I)·poly(C) agarose after the conversion of ATP to ADP using hexokinase/glucose (Bruchelt *et al.*, 1989).
3. RNase L radiobinding assay (Knight *et al.*, 1980). Unknown 2-5A solutions from crude reaction mixtures (Knight *et al.*, 1981) or after HPLC purification (Brown *et al.*, 1981) can compete with radiolabelled 2-5A for RNase L binding.
4. Antibody binding
 - A. Radioimmunoassay. The minimal structure recognized is -A2'pA2'pA- (Knight *et al.*, 1981), -A2'pA- (Cailla *et al.*, 1982), or (pp)pA2'pA- (Sawai *et al.*, 1985a).
 - B. Enzyme immunoassay. The minimal structure recognized is -A2'pA- (Nicolas *et al.*, 1987).
 - C. ELISA. The minimal structure recognized is -pA2'pA- (Johnston *et al.*, 1983).
5. Spectrophotometry. This pyrophosphate assay measures NADPH through the use of UDP-glc pyrophosphorylase, phosphoglucomutase, and G6PD (Justesen and Kjeldegaard, 1992).

synthetase adsorbed to poly(I)·poly(C) has been shown to be more stable (Samanta *et al.*, 1980) and to demonstrate a linear synthesis of 2-5A for a longer time (Hovanessian *et al.*, 1981a) than enzyme in solution. The synthesis has been commonly terminated by removal of the reaction mixture from the support, or if the reaction is done in solution, denaturation of the enzyme with heat followed by centrifugation.

Initial efforts to determine the concentration of 2-5A utilized its known biological activity (Table 7). That is, the assay took advantage of the ability of 2-5A to inhibit protein synthesis in cell-free systems or examined RNA cleavage by 2-5A-activated RNase L. However, the relative insensitivity, the inherent variability involved in using a complex biological system as an assay method, and the unsuitability of these techniques for routine analysis render them of limited interest.

A number of useful assays exist that utilize the incorporation of radiolabelled ATP into 2-5A by the 2-5A synthetase reaction mixture. The mixture of radiolabelled 2-5As, or their core (5'-unphosphorylated) derivatives (the latter obtained after treatment with alkaline phosphatase), can be

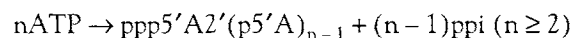
separated by any of several methods. Ion-exchange methods utilizing alumina columns or DEAE on sephadex or paper commonly have been used. Reverse-phase thin-layer chromatography (TLC) techniques also have been useful in visualizing the 2-5A molecules generated. In addition, gel and paper electrophoresis, as well as capillary isotachophoretic methods, have been adapted for quantitation of the reaction products. High performance liquid chromatography (HPLC) remains a powerful technique, as it may be used for preparative, as well as analytical, separations of the radiolabelled 2-5As.

2-5A oligonucleotide assays that rely on the high-affinity binding of 2-5A to RNase L (Knight *et al.*, 1980) or to monoclonal antibodies have the advantage of high specificity and sensitivity (Table 7). RNase L from crude cell extracts binds ppp5'A2'pA5'A2'p5'A3'[³²P]pCp (2-5A[³²P]pCp) strongly. Inhibition of binding of this probe takes place with nanomolar concentrations of unlabelled 2-5A. Similarly, labelled 2-5A (2-5A[³²P]pCp) (Knight *et al.*, 1980) or [¹²⁵I]succinyl 2-5A (Cailla *et al.*, 1982) binding to antibodies can be competed off by nanomolar concentrations of unlabelled 2-5A.

However, with the antibody assays, cross-reactivity with 3',5'-oligoadenylates, which may be present in the millimolar range, can be problematic. The latter may have to be removed before the assay can be performed. Finally, enzyme immunoassays, ELISAs, and coupled spectrophotometric assays have the advantage that no radiolabelled 2-5A mixtures or radiolabelled probes need to be generated (Table 7).

3.6. Enzymology

2-5A synthetase catalyzes the following reaction:



In addition to catalyzing the polymerization of ATP to 2',5'-oligoadenylate, the enzyme may also function as a 2',5'-nucleotidyltransferase (Ball and White, 1979a; Johnston *et al.*, 1980a; Ferbus *et al.*, 1981). A single addition of any of the different ribo- and deoxyribonucleotide triphosphates onto the 2'-OH can occur (Hughes *et al.*, 1983), although some phosphorothioate ATP analogues (Kariko *et al.*, 1987a) do not seem to act as chain terminators. 2-5A synthetase can also elongate various oligonucleotides (as primers), which

terminate in adenosine and in which a free 2'-OH exists (Ball and White, 1979a; Minks *et al.*, 1980b; Ferbus *et al.*, 1981). Since 2-5A synthetase 2'-adenylates both NAD⁺ and Ap₄A, thereby inactivating them, the initial speculation was that 2-5A synthetase played a role in cellular metabolism by this route; however, to date, these 2'-adenylated intermediates have not been identified in cells using the radiobinding assay discussed above (Cayley and Kerr, 1982).

2-5A synthetase has been purified to homogeneity from human HeLa and Namalva cells, mouse L and EAT cell lines, as well as from rabbit reticulocytes (Table 8). Common purification steps have included ammonium sulfate or methanol precipitation and DEAE-cellulose chromatography (Wells *et al.*, 1984; Dougherty *et al.*, 1980; Ball, 1980). The most convenient overall schemes did not require ultracentrifugation (Yang *et al.*, 1981). Using p69 and p100 immunoaffinity columns, Marie *et al.* (1989) purified these two isoforms to homogeneity. A necessary purification step has been chromatography on poly(rI)·poly(rC)-cellulose or poly(rI)·poly(rC)-agarose, as the enzyme is unstable in the absence of dsRNA (Rovnak and Ranu, 1987). Yang *et al.* (1981) found that ATP, dsRNA, BSA, and nonionic deter-

TABLE 8. Molecular Weights of 2-5A Synthetase mRNAs and Protein Isolated from Different Sources

Species	Cell type	mRNA (kDa)	Protein MW (kDa)	Reference	
Human	HeLa		80, GG	Yang <i>et al.</i> , 1981	
			100, 105, SDS-PAGE	Wells <i>et al.</i> , 1984	
			30 and 70, GF	Revel <i>et al.</i> , 1981	
			33 and 110, GF	Ilson <i>et al.</i> , 1986	
			100, SDS-PAGE	Raber <i>et al.</i> , 1991	
	Namalva	SV80	1.6, 1.8, 3.6	30, GF	Revel <i>et al.</i> , 1981
		Namalva	1.8		Merlin <i>et al.</i> , 1983
		SV80	1.6, 1.8, 3.6		Benech <i>et al.</i> , 1985a
		FS11	1.6, 1.8, 3.6		
		WISH	1.6, 3.6		
		U937	1.6, 3.6		
		Namalva, Daudi	1.8		
		R17 (IFN- α -resistant Daudi)	1.8		
		CEMT	1.6, 1.8, 3.6		Benech <i>et al.</i> , 1985b
		SV80, Namalva, HeLa	1.6, 1.8		Chebath <i>et al.</i> , 1985
T98G, Daudi	1.7		Saunders <i>et al.</i> , 1985		
Mouse	HeLa		1.5, 1.7, 2.5, 3.5		
		KB, FL	1.6-1.8, 3.2, 3.6		
	L		90, GF	Shiojiri <i>et al.</i> , 1985	
	EAT		85, GG	Revel <i>et al.</i> , 1981	
			105, SDS-PAGE	Dougherty <i>et al.</i> , 1980	
	EAT S30	1.5	85-100, GG	St. Laurent <i>et al.</i> , 1983	
Rabbit	Reticulocyte	EAT Nuclei	20-30, GG		
		3T3	105, SDS-PAGE	Raber <i>et al.</i> , 1991	
			106, SDS-PAGE	Wells <i>et al.</i> , 1984	
Pig	Spleen		110, SDS-PAGE	Rovnak and Ranu, 1987	
			100, GF	Shimizu and Sokawa, 1983	
			150, GG	Shimizu <i>et al.</i> , 1984	
Chicken	Embryo fibroblasts		20-40, GG		
			50-60, GF	Ball and White, 1979b	
			56, SDS-PAGE	Ball, 1980	

GF, gel filtration; GG, glycerol gradient density centrifugation.

gents such as Triton X-100 or Nonidet P-40 stabilized the enzyme. The K_m of the 2-5A synthetase was ~ 2 mM; >90% of ATP added can be utilized, with the optimal Mg^{2+} concentration of 16 mM. The consensus is that mode of chain elongation is nonprocessive (Johnston *et al.*, 1980b; Justesen *et al.*, 1980; Imai *et al.*, 1982b; but see Minks *et al.*, 1980a): intermediates dissociate from the enzyme before addition of another nucleotide unit. Experiments to confirm this with the purified 2-5A synthetase isoforms have not been performed.

The human p40-46 2-5A synthetase (accession numbers X04371 and M25352) and p69 2-5A synthetase (accession number M87284) have been cloned (Wathelet *et al.*, 1986; Marie and Hovanessian, 1992); however, human p100 has not been reported yet to be cloned. The p40 and p46 2-5A synthetases are coded for by 1.6 and 1.8 kb mRNAs, which are derived from the same gene through cell type-specific differential splicing between the 7th and an additional 8th exon of this gene (Benech *et al.*, 1985a), which is localized to chromosome 12 (Williams *et al.*, 1986). The 1.6 kb RNA codes for a 364 amino acid C-terminal hydrophobic protein, while the 1.8 kb RNA codes for a 400 amino acid C-terminal acidic protein (Benech *et al.*, 1985b; Saunders *et al.*, 1985). The cDNAs encoding both 1.6 and 1.8 kb mRNAs have been expressed in *E. coli* under a *trp-lac* promoter (Mory *et al.*, 1989).

Two p69 2-5A synthetase full-length cDNAs of 5.6 and 3.1 kb encode open reading frames (ORFs) that are predicted to produce a protein with the first 683 amino acids identical, with an extension of 4 or 44 amino acids (Marie and Hovanessian, 1992). When translated, these encoded proteins of 69 and 71 kDa migrated with authentic p69 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and produced 2-5A in the presence of ATP and dsRNA. The C-terminal acidic p69 and C-terminal hydrophobic p71 are seen as a broad band sometimes visible as a doublet on SDS-PAGE (Hovanessian *et al.*, 1987). The p69 2-5A synthetase contained two homologous subdomains, each containing an ATP binding motif (Gly-gly-Ser-X-gly/Ala-Lys/Arg), as well as a dsRNA-binding domain. In addition, p40 and p46 contained only one of each of these domains. On the basis of gel-filtration experiments (Marie *et al.*, 1990a,b) and the former evidence, it is likely that p40 and p46, as well as p69 and p71, probably exist as a tetramer and dimer, respectively, which possess two 2-5A catalytic domains. On the other hand, the p100 2-5A synthetase probably exists only as a monomer (Marie *et al.*, 1990b), which is consistent with its observed role in the production of 2-5A dimers (Hovanessian *et al.*, 1988). It is also tempting to speculate that the p69-p71 gene may have evolved from the ancestral fusion of two p40-p46 genes.

The four forms of 2-5A synthetase (p40, p46, p69, p100) have been isolated from a variety of human cell lines and from PBMCs; however, there were a number of biochemical and functional differences between them. Although any of the isoforms have been induced by IFN- α , - β or - γ , provided that cell type was responsive to that type of IFN,

there have been cell-type (Marie *et al.*, 1990b) and patient-specific (Witt *et al.*, 1993a) differences in the dose-response and kinetics of the 2-5A synthetase isoform synthesis. Differences in subcellular localization also occurred. Both p69 and p100 were localized in the cytoplasm, but p69 was myristoylated and was found in closer association with the nuclear membrane (Marie *et al.*, 1990b). The p69 was present in cell membranes as well as the microsomal pellet, while p100 was found only in microsomes (Hovanessian *et al.*, 1987). The p46 and p46 2-5A synthetases were localized primarily in nuclei (Chebath *et al.*, 1987a). The p69 2-5A synthetase had a higher K_m for ATP, required less dsRNA to manufacture 2-5A, required a more acidic pH, and produced longer 2-5A oligomers. In addition, maximal synthesis of 2-5A occurs earlier in response to IFN than does p100 (Hovanessian *et al.*, 1988). Although the four isoforms were antigenically dissimilar, they did share common epitopes (Chebath *et al.*, 1987a).

Binding of a Type I IFN led to a tyrosine phosphorylation of the human IFN- α receptor peptide (IFNABR- α) and an activation by phosphorylation of the latent transcription factor (ISGF3/E) that can bind to the ISRE (see Pellegrini and Schindler, 1993).

The 40 kDa isoform of 2-5A synthetase was expressed as a glutathione S-transferase fusion protein in *E. coli* (Kon and Suhadolnik, 1996). Covalent linkage of a 8-azido- $[^{32}P]$ ATP photoprobe to 2-5 synthetase, tryptic digestion of the bioconjugate, and isolation of the labeled peptide by metal (Al^{+3}) chelate chromatography revealed a pentapeptide to be the putative ATP binding site. The sequence was $D_{196}FLKQ_{200}$ where the photoprobe modified lysine 199. Site-directed mutagenesis of this lysine confirmed the results.

3.7. Structure-Activity Relationships for Double-Stranded RNA Binding

The binding site for dsRNA to 2-5A synthetase isoforms has not been well defined. Ghosh *et al.* (1991) investigated a small mouse 2-5A synthetase and found that a linear region between residues 104–158 at its amino terminal domain was necessary for dsRNA binding. This sequence showed some homology with other mouse and rat 2-5A synthetases (Truve *et al.*, 1994b), and has been postulated to be the dsRNA binding site. Though human p40 (Wathelet *et al.*, 1986) showed some homology in this region, there was little homology to human p69 (Marie and Hovanessian, 1992), while sequences beginning at residues 201 in p40 and at residues 192 and 535 in p69 contained 7–9 lysine or arginine residues and were more strictly conserved. Moreover, while the dsRNA-binding domain of the dsRNA-activated protein kinase PKR is shared by dsRNA binding proteins from a number of organisms (St. Johnston *et al.*, 1992), 2-5A synthetases appear to belong to a completely different family of dsRNA binding proteins.

Maximal 2-5A synthetase activity required the presence of 65–80 bp long dsRNA (Minks *et al.*, 1979a,b,c; Samanta *et al.*, 1980). If dsRNA was removed from the 2-5A syn-

thetase reaction by RNase III treatment, the reaction stopped (Minks *et al.*, 1980b). The influence of dsRNA structure on activation has been extensively explored. The proteins p69 and p100, purified by immunoaffinity chromatography, were activated by poly(I)·poly(C), as well as poly(A)·poly(U), though the latter homopolymer-activated p69 somewhat less well than the former (Marie *et al.*, 1990a). Single-stranded RNAs (ssRNAs) could not activate 2-5A synthetase in this study, although both isoforms could bind to ssRNA-sepharose. While bound, the enzyme could become activated by poly(I)·poly(C), and on this basis, the presence of multiple polynucleotide binding sites was postulated (Marie *et al.*, 1990a). The partly dsRNA adenoviral VAI RNA can bind to both the PKR protein and the 2-5A synthetase, but only 2-5A synthetase is activated by VAI RNA (Desai *et al.*, 1995).

The misaligned dsRNA poly(I)·poly(C₁₂U) (Ampligen) has also been shown to be a potent activator of p69 and p100 2-5A synthetase both *in vitro* and *in vivo* (Carter *et al.*, 1987; Li *et al.*, 1990; Ushijima *et al.*, 1993; Hovanessian *et al.*, 1988). Poly(I)·poly(C₁₂U) was also less toxic, less pyrogenic, and less antigenic than is poly(I)·poly(C) (Hearl and Johnston, 1986). Synthetic heteropolymeric dsRNA has also been prepared and was found to activate 2-5A synthetase (Haines *et al.*, 1992). It possessed restricted biological activity, however, being unable to inhibit the growth of A1698 cells *in vitro* or induce IFN- β or IL-1 α gene expression.

Structure-activity studies have demonstrated that neither ssRNA, triple-stranded RNA, nor complete 2'-OH substitution of poly(I)·poly(C) with H, OCH₃, N₃, F, or Cl resulted in activation (Johnston *et al.*, 1980a; Baglioni *et al.*, 1981b; Torrence *et al.*, 1981b; Torrence and Friedman, 1979). 2-5A synthetase, however, was more tolerant of some 2'-O methyl substitution than was PKR (Minks *et al.*, 1980b).

Natural dsRNAs from phage f_2 , killer yeast or *Penicillium chrysogenum* have been found to be good 2-5A synthetase activators. Adenoviral VAI RNA, which is highly structured in solution, can bind to and activate 2-5A synthetase as a consequence of its two long, imperfectly paired stem structures (Desai *et al.*, 1995; Matthews and Shenk, 1991). When the imperfect pairing was repaired, 2-5A synthetase was activated to an even greater degree. EMCV RNA was also found bound to p40 2-5A synthetase after immunoprecipitation and the p40 was partially activated, even without the addition of dsRNA, while p40 precipitated from uninfected cells was not (Gribaudo *et al.*, 1991). The HIV transactivating region (TAR) was able to bind and to activate 2-5A synthetase *in vitro* (SenGupta and Silverman, 1989; Maitra *et al.*, 1994). Further, this effect of TAR on 2-5A synthetase activation was abolished by the Tat protein (Schroder *et al.*, 1990c; Silverman and SenGupta, 1990). Heterogeneous nuclear RNA (hnRNA) from PBMCs from patients with hairy cell leukemia and from HeLa cells activated 2-5A synthetase, suggesting a possible role in hnRNA processing (Nilsen *et al.*, 1982b; Hubbell *et al.*, 1991). 2-5A synthetase has also been found to be associated with 60S spliceosomes

and could be immunoprecipitated with anti-Sm antibodies. 2-5A synthetase antibodies were also found to inhibit the splicing reaction *in vitro* and to result in deficient splicing activity when added to HeLa cell nuclear extract (Sperling *et al.*, 1991).

Remarkably, 2-5A synthetase from lymphocytes of autoimmune BB rats was not sensitive to stimulation by dsRNA (Bonnievie-Nielsen *et al.*, 1991b).

4. RIBONUCLEASE L, THE 2-5A-DEPENDENT NUCLEASE

4.1. Occurrence

The overwhelming majority of data is consistent with the view that RNase L is present in avian, reptilian, and mammalian species, but not in insects, fish, plants, or bacteria. Reptiles and avian species have been shown to possess 2-5A binding activity (presumably RNase L) in a number of studies (Ball and White, 1978a,b; Cayley *et al.*, 1982b). In fact, liver, kidney, and cardiac tissues from a number of reptilian species have been shown to be high in 2-5A binding activity (Cayley *et al.*, 1982b).

Murine and rabbit tissues in which RNase L has been found are listed in Table 9. The enzyme also is found in a large number of human cell types in the absence of IFN treatment. In one study, murine spleen tissue extracts had the highest levels of RNase L, while liver extracts had the lowest levels, as measured by the radiobinding assay (Floyd-Smith and Denton, 1988a). Lung and kidney extracts had moderate levels. While IFN- β did not affect the observed basal level of RNase L present in tissues, rabbit anti-mouse IFN- α/β did decrease levels of RNase L in spleen, suggesting that endogenous IFN- α/β was responsible for the maintenance of RNase L levels in that organ *in vivo*. In mice, RNase L levels are highest in the spleen, lung, liver, and kidney at 5 days of age and then decrease with age, except in the spleen where it remains high until adulthood (Floyd-Smith and Denton, 1988b). This may have relevance to the participation of the RNase L system in certain age-specific viral diseases.

The majority of studies have identified a nuclear and cytoplasmic localization for RNase L (Nilsen *et al.*, 1982c; Ba-

TABLE 9. Mammalian Cellular Distribution of RNase L

Mammalian cell type	Reference
Rabbit (liver, kidney, spleen, reticulocytes)	Williams <i>et al.</i> , 1979a; Nilsen <i>et al.</i> , 1981; Krause and Silverman, 1993
Mouse (liver, kidney, lung, intestine, spleen)	Nilsen <i>et al.</i> , 1981; Floyd-Smith and Denton, 1988a,b; Silverman <i>et al.</i> , 1988; Bayard <i>et al.</i> , 1994
Human lymphocytes	Nilsen <i>et al.</i> , 1981
Human colonic mucosa	Wang <i>et al.</i> , 1995
Human kidney	Bayard and Gabrion, 1993

yard and Gabrion, 1993; Schroder *et al.*, 1989). The enzyme was associated with polysomes in the cytoplasm (Salehzada *et al.*, 1991) and with nuclear matrices in the nucleus (Schroder *et al.*, 1989).

Basal levels of RNase L exist in a wide variety of differentiated cells types at low, constant levels; however, in some cell types, RNase L may be highly regulated. In murine JLS-V9R cells, RNase L levels increased up to 20-fold with IFN treatment, a response that was blocked by treatment with actinomycin D, suggesting transcriptional involvement (Jacobsen *et al.*, 1983a,b). Furthermore, a 10-fold increase in RNase L levels could be observed in this cell type during growth arrest by confluency. In NIH 3T3 cells, growth arrest by confluency caused a 6-fold induction of RNase L levels that could be increased still further by IFN treatment (Krause *et al.*, 1985a,b). RNase L mRNA levels were increased 3-fold in murine L929 cells as a response to IFN treatment and were unaffected by cycloheximide (Zhou *et al.*, 1993).

There are a number of indications that RNase L functions in the control of cell growth and differentiation. As documented above, RNase L and 2-5A synthetase levels often were found to be elevated during cell differentiation, as well as in growth-arrested cells. In addition, introduction of 2-5A into cells has the effect of impairing cell growth (Sen and Lengyel, 1992; Zhou *et al.*, 1993). Krause *et al.* (1985a,b) demonstrated differential regulation of RNase L in various cell lines in response to induced differentiation or IFN treatment, as measured by the breakdown of rRNA caused by introduction of ppp5'(A2'p5')₂A into the cells. RNase L induction by IFN was demonstrated for three differentiated cell types (F9 clone 9, PYS, and PSA 5E); however, in three undifferentiated cell lines (F9, PC13 clone 5, and Nulli 2A), there was little RNase L with or without IFN treatment. In addition, during induced differentiation of PC13 clone 5 cells, there was a concomitant induction of RNase L. Paradoxically, a recent study suggests that RNase L levels may be increased up to 10-fold in rapidly dividing tissue obtained from colorectal tumors with respect to normal colonic mucosa (Wang *et al.*, 1995). The increased RNase L levels were also found to occur early during colorectal tumorigenesis.

The effects of 2.45-GHz microwave radiation on 2-5A synthetase and RNase L levels were investigated (Krause *et al.*, 1991). Although no differences in 2-5A synthetase levels were found, RNase L was induced as measured by 2-5A binding.

4.2. Genomics

The gene for RNase L is known as RNS4, as it was the fourth RNase to be mapped in the human genome (Squire *et al.*, 1994). The regional assignment was made using fluorescence *in situ* hybridization using a RNase L genomic clone isolated from a human placenta cosmid library (Zhou *et al.*, 1993). Positive hybridization signals were found on 1q25 in all of 30 metaphase spreads. Since the genes for 2-5A

synthetase have been mapped to chromosomes 11 and 12, genes for the 2-5A system do not form a gene cluster in the human genome. There was no evidence that RNS4 defects are directly linked to human disease; however, there are a number of abnormalities that co-localize with 1q defects. The Charcot-Marie-Tooth disease (CMT1B) locus has been localized to 1q21.2 to 1q25 (Lebo *et al.*, 1991). Multiple congenital anomalies and developmental delays were found in one patient with an interstitial deletion between 1q23 and 1q25 (Franco *et al.*, 1991). More significantly, a number of human cancers have been found to be associated with 1q abnormalities. These include gastric adenocarcinomas (Sano *et al.*, 1991), oral squamous cell carcinomas (Kerr and Brown, 1978), and breast cancer (Chen *et al.*, 1989; Pathak *et al.*, 1990). Because of the negative growth regulatory function of RNase L, it may be considered a candidate for a tumor-suppressor gene.

4.3. RNase L Cloning, Purification, and Properties

Human and murine RNases L have been successfully cloned. Murine L₉₂₉ cells treated with IFN/cycloheximide and then medium alone were chosen as the initial source of mRNA (Zhou *et al.*, 1993) because of relatively abundant basal levels of RNase L. The cDNA library was screened using a bromine substituted [³²P]2-5A probe (Nolan-Sorden *et al.*, 1990). *In vitro* translation in wheat germ extract with subsequent characterization using the aforementioned probe and proteolytic degradation analysis revealed that the isolated murine RNase L was truncated with an apparent molecular mass of 74 kDa. The sequence of full-length human RNase L was obtained from a composite cDNA(HZB1)/genomic construct. To obtain the cDNA portion, a human kidney cDNA library was screened with the previously obtained radiolabeled murine RNase L cDNA. The sequence resulted in an ORF encoding a protein of 83,539 Da. After *in vitro* translation in a rabbit reticulocyte lysate that had been depleted of endogenous RNase L, core-cellulose-based assays with the enzyme revealed the degradation of poly(U) with a half-life of 10 min; using the same conditions, no poly(C) degradation occurred. Localization of the 2-5A-binding domain was facilitated using a C-terminus truncated murine RNase L that possessed no catalytic activity. Deletion analysis, as well as site-directed mutagenesis, of the human construct localized the 2-5A-binding domain to between amino acids 218 and 294 at the site of two putative P-loop motifs. Additionally, a Zn-finger was proposed, although the putative homologous region (amino acids 394-444) is atypical of classical Zn fingers, and the human and mouse sequences differ in the positions of the cysteines.

An independent analysis of the sequence of RNase L (Bork and Sander, 1993) found that the N-terminal 330 residues contained 9 ankyrin (ANK) repeats, with an average length of 33 residues with 35% sequence homology to human erythrocyte ANK. ANK repeats occur as consecutive copies in a large number of functionally variable proteins, and have been shown to mediate protein-protein and

protein-DNA interactions. Protein kinase domains have been identified following the ANK repeats. The previous identification of a P-loop motif has been challenged by this group (Bork and Sander, 1993). Nevertheless, the 7th and 8th ANK repeat contains the likely 2-5A binding site, whether or not in association with two P-loops, and the protein kinase homologous domains probably contain the ATP binding site, with domains necessary for catalytic activity residing in the C-terminal portion of the enzyme.

Dong and Silverman (1997) have performed progressive truncation studies from either terminus of RNase L, and have developed a bipartite model for the structure of RNase L in which the regulatory functions were assigned to the N-terminal half of the enzyme and the catalytic function was assigned to the C-terminal half. In the process of these studies, Dong and Silverman were able to produce, by deletion of 335 amino acids from the N-terminus, a constitutively active ribonuclease with about 6-fold reduced activity compared with wild-type enzyme.

Recombinant RNase L recently has been expressed in a baculovirus system and purified to reported homogeneity (Dong *et al.*, 1994). The human RNase L cDNA was subcloned into the BacPAK6 baculovirus vector and plaques containing recombinant virus selected based on Southern analysis. Expression was accomplished in SF21 cells using a multiplicity of infection of 10 for 72 hr. After preparation of cell extract, FPLC chromatography using successive Blue Sepharose CL-6B, Mono Q, and Superose-12 columns yielded apparently pure enzyme.

This recombinant enzyme was evaluated for its activator requirements. While the dimer pppA2'p5'A and 3',5'-phosphodiester-linked oligoadenylates such as ppp5'(A3'p5')₂A completely failed to activate RNase L, and while 2'-5' linked core oligoadenylates such as A(2'p5')₂A had greatly reduced ability to activate the enzyme, the longer 2-5A oligomers, ppp5'(A2'p5')₂A, ppp5'(A2'p5')₃A, as well as the monophosphate p5'(A2'p5')₃A all maximally activated at 1 nM. After extensive dialysis to remove divalent cations, the effects on enzyme activity, as measured by the poly(U) degradation assay, also were determined. Although recombinant RNase L had some residual ability to cleave poly(U) even in the complete absence of metal ions, addition of ATP plus Mg cation or, to a slightly lesser extent, ATP plus Mn cation significantly stimulated RNase L activity. Addition of Ca ions alone was inhibitory, as was the addition of Zn ion, even in the presence of ATP. At a 1:1 molar ratio of 2-5A to RNase L, as determined by glycerol gradient centrifugation, chemical cross-linking, and gel filtration, complete conversion to a homodimeric form from a monomer was observed. In addition, core oligoadenylates were ineffective at promotion of dimerization. This may indicate the presence of a shared catalytic site for RNA degradation in RNase L. While poly(rG), poly(rC), poly(dA), and poly(dT) were not cleaved by the recombinant enzyme, poly(rA) was degraded somewhat, and poly(rU) was degraded very efficiently, to fragments ranging in size from between 5 and 22 nucleotides in length.

It was first observed that an irreversible loss of RNase L activity was caused by gel filtration through a Sephadex G-25 column, and that this could be prevented by addition of ATP or ADP to the buffer (Wreschner *et al.*, 1982). In more recent work, purified mouse and human RNase L showed 2-fold stimulatory effects of ATP on RNase L activity, but only in the presence of 2-5A (Silverman *et al.*, 1988; Dong *et al.*, 1994). Earlier results notwithstanding, if ATP was omitted during some purification steps, RNase L activity was fully recovered. This observation has been repeated in the authors' laboratory (M. Player, E. Wondrak and P. F. Torrence, unpublished observations). RNase L activation also requires the presence of Mg²⁺, although Mn²⁺ or Ca²⁺ will also function to support catalytic activity. A minimal level of activity remains in the absence of divalent cations (Dong *et al.*, 1994). A reducing agent such as 2-mercaptoethanol or dithiothreitol is also necessary to maintain the binding activity of the ribonuclease (Dong *et al.*, 1994; Bayard *et al.*, 1994), although no detailed studies on this are available.

RNase L was found to exist in the monomeric state with a molecular mass of approximately 80 kDa in rabbit reticulocytes (Wreschner *et al.*, 1982), CEM and Jurkat cells (Bayard and Gabrion, 1993), mouse liver and EAT cells (Silverman *et al.*, 1988; Floyd-Smith *et al.*, 1982), as well as HeLa cells and mouse thymoma W7 cells (Nilsen *et al.*, 1981). However, murine EAT cells and mouse splenocytes have also been found to contain a dimer of approximately twice this weight (Slattery *et al.*, 1979; Bayard *et al.*, 1994).

In an additional study, murine Ehrlich ascites tumor cell-derived RNase L was reported to behave as a 185 kDa species by gel filtration, while SDS-PAGE analysis of an affinity-labeled RNase L gave a result of 80 kDa (Bisbal *et al.*, 1989). However, these latter authors found another 40 kDa species that bore some similarity to RNase L by partial proteolysis. Protease inhibitors were reported not to alter the levels of the 40 kDa protein, and its significance remains unknown. It has also been proposed that RNase L consists of a heterodimer: a regulatory subunit that binds 2-5A and a catalytic subunit to which RNA binds (Salehzada *et al.*, 1993). More recent work, based on chemical cross-linking, gel filtration, and glycerol gradient sedimentation, demonstrated that the enzyme is a homodimer in the presence of a molar equivalent of 2-5A and a monomer in its absence (Dong *et al.*, 1994; Cole *et al.*, 1996). 2-5A oligoadenylates, which caused dimerization of RNase L, usually activated it well, while core (non-5'-phosphorylated) oligoadenylates, which were not capable of activating RNase L, were similarly inefficient at dimerizing it (Dong *et al.*, 1994).

Dimerization of RNase L has been studied by equilibrium sedimentation techniques. The enzyme was found not to dimerize in the presence of a synthetic oligoribonucleotide substrate or in the presence of ATP alone (Cole *et al.*, 1996). However, in the presence of saturating (2'-5')pA₃, the enzyme was fully dimerized at protein concentrations as low as 100 nM. In the absence of activator, no dimerization was observed at protein concentrations as high as 18 mM.

Evaluation of the kinetics of cleavage of PKR mRNA in the context of 2-5A antisense chimeras has been performed (Maitra *et al.*, 1995). The 2-5A antisense chimera against PKR mRNA, as well as 2-5A tetramer, were found to be 20- and 40-fold more active, respectively, than a 2-5A antisense chimera in which the antisense sequence was not complementary to the PKR mRNA target. In addition, k_{cat} was found to be 5–10 sec^{-1} , implying that each molecule of RNase L was able to catalyze the cleavage of multiple mRNA strands. The sites of cleavage were also mapped to discrete sites along the PKR mRNA using primer extension analysis. The recognized preference for cleavage 3' of UpNp was altered by this approach, and a clear preference for specific bases was not observed.

Although classical methods of assay rely on cleavage of radiolabelled poly(U) (Silverman, 1985) or on cleavage of ribosomal RNA (rRNA) (Kariko *et al.*, 1987a,b), a more recent method utilizes cleavage of a radiolabelled oligoribonucleotide (Carroll *et al.*, 1996). Since RNase L cleaves preferentially after UU and UA sequences (Wreschner *et al.*, 1981b), the sequence chosen for kinetic analysis of RNase L was [^{32}P]5'-pC₁₁UUC₇. Comparison of the primary cleavage product with cleavage products resulting from RNase PhyM and from base-catalyzed hydrolysis confirms that [^{32}P]5'-pC₁₁UUp is the primary cleavage product.

When RNase L was added to a reaction mixture containing substrate and (2-5)pA₃, an initial lag in product formation occurred. This could be alleviated by a 30-min incubation of RNase L with (2-5)pA₃ at 0°C. The rate of cleavage was linear between 100 and 600 pM, using RNase L preactivated with 800 nM (2-5)pA₃. The rate of cleavage was half-maximal at 1 nM (2-5)pA₃, and attained maximal velocity at 1 nM (2-5)pA₃ and above. Longer oligoribonucleotides were cleaved more efficiently; for instance, 5'-pC₅UUC₄ is cleaved 3-fold less efficiently (k_{cat}/K_m) than 5'-pC₁₁UUC₇, primarily due to an increase in K_m . In addition, RNase L catalyzed the cleavage of substrates containing 2 or 3 sequential U's with a 20- and 50-fold faster rate than that with which it catalyzed the cleavage of a substrate containing a single U (Carroll *et al.*, 1996).

The 2-5A system and the RNase L are rapidly evolving in complexity. Salehzada *et al.* (1993) obtained a monoclonal antibody by immunizing BALB/c mice with Daudi cell proteins partially purified on a 2-5A-Sepharose column. This antibody, together with 2-5A binding experiments, enabled these investigators to distinguish between two different proteins in Daudi cell extracts: a 2-5A-binding protein and an RNA binding protein. The investigators proposed that RNase L is actually a complex of two separate subunits: an 80 kDa RNA binding protein (the catalytic subunit) and an 80 kDa 2-5A binding protein (regulatory subunit). The possible relationship of this relationship, if any, to the above described dimerization, is unknown as yet.

In a separate study, Bayard *et al.* (1994) employed tetrameric ppp(5'A₂'p)₃A, periodate-oxidized and then covalently bound to hexanoic hydrazide agarose, to affinity purify RNase L from 100,000 g supernatants of mouse

spleen. Highly purified enzyme, >80% homogenous, could be eluted from such an affinity column with pH 2.7 glycine buffers in the presence of Triton-X. When the purified nuclease was stabilized by affinity labeling with 2-5A, it migrated as a 160 kDa protein, but as an 80 kDa protein in denaturing SDS-PAGE conditions. Similar studies with cellular extracts implied that the enzyme was comprised of 80% 160 kDa and 20% 80 kDa species. In the absence of 2-mercaptoethanol, the apparently dimeric 160 kDa species bound 2-5A only minimally. The addition of 2-mercaptoethanol, or, surprisingly, poly(U), could maximize 2-5A binding. The eluted 160 kDa nuclease showed residual activity even in the absence of 2-5A. These findings led Bayard *et al.* (1994) to suggest that the poly(U) substrate induced a conformational change in RNase L, and that this change facilitated 2-5A binding. Bayard *et al.* (1994) also postulated the loss of a regulatory protein during purification in order to account for the low level of non-2-5A-dependent nuclease activity.

Yet another chapter in the complexity of the 2-5A system was added through the findings of Bisbal *et al.* (1995), who isolated a polypeptide inhibitor (termed RLI) of the 2-5A system based on their screening of an expression library assayed by binding to radioactive 2-5ApCp. This protein was proposed as a regulator that would inhibit the binding of 2-5A to RNase L, thereby blocking activation of RNase L and its nuclease activity. Although RLI had a poor affinity for RNase L, it may associate directly, but noncovalently, with the enzyme to alter its activation potential by 2-5A. Over-expression of RLI in HeLa cells partly antagonized the antipicornaviral effects of IFN.

In essence, the present state of affairs in regard to RNase L's functional catalytic structure may be expressed by two different scenarios. On the one hand, as suggested by the work of Dong *et al.* (1994) and Cole *et al.* (1996), RNase L appears as an 83 kDa protein that dimerizes in the presence of a 2-5A activator, perhaps to give the actual catalytic conformation. On the other hand, as represented by the data of Bayard *et al.* (1994) and Salehzada *et al.* (1993), RNase L may consist of two different 80 kDa proteins, one that binds 2-5A and another that binds RNA substrate. The active enzyme could then be inhibited by yet another protein that binds RNase L, but not 2-5A.

4.4. Cleavage Specificity

A critical question is whether or not the 2-5A system might differentiate between RNA substrates, i.e., viral vs. cellular, in the intact host cell. Partially purified RNase L from murine EAT cells was found to degrade only poly(U) of the four homopolymers: poly(U), poly(C), poly(G), and poly(A) (Floyd-Smith *et al.*, 1981). With a 5'-labelled phage T7-specific RNA segment, frequent cuts were found after UA, UG, and UU, with minor cleavages after CA and rare cleavages after AC and CG (Floyd-Smith *et al.*, 1981). RNase L isolated from rabbit reticulocytes, human lymphoblastoid cells, and murine EAT cells cleaved 3'-labelled viral and

18S rRNA substrates after some, but not all, UU and UA sequences, possibly as a result of secondary structure (Wreschner *et al.*, 1981a,b). Generally, then, RNase L cleavage occurred 3' to UpN3'p terminated products (Wreschner *et al.*, 1981a,b). Cleavage experiments with purified recombinant human RNase L yielded similar results, with the exception that inefficient cleavage of poly(A) was observed (Dong *et al.*, 1994). The existence of cuts in predominantly single-stranded regions of phage R17 RNA (Floyd-Smith *et al.*, 1981) and the reported absence of cleavage of poly(A).poly(U) (Nilsen *et al.*, 1980) have been established, but the dependence of sites and extent of cleavage on RNA secondary structure is poorly understood. In addition, the degradation of poly(U) was observed to generate discrete fragments or about 5–22 nucleotides in length as end products for unknown reasons (Dong *et al.*, 1994).

Cleavage of specific types of RNA could occur in the intact host cell through any of several mechanisms. Compartmentalization of 2-5A synthetase or RNase L to areas within the cell could result in localization of either 2-5A synthesis or action. Several findings point to this as a possibility. The replicative intermediates of certain viruses contain dsRNA linked to ssRNA, and ssRNA linked to dsRNA has been found to be degraded faster than ssRNA alone in extracts of IFN-treated cells, possibly as a result of localized 2-5A production (Nilsen and Baglioni, 1979). Nuclear extracts of HeLa cells were found to contain nanomolar quantities of 2-5A and an activity that bound 2-5A with an affinity similar to that of RNase L. The nucleic acid, hnRNA, which contains duplex structures, was found both to promote the synthesis of 2-5A and to be preferentially cleaved in IFN-treated cell extracts (Nilsen *et al.*, 1982b). Nascent reovirus RNA prepared in transcription reactions from purified virions was degraded in preference to mRNA in a cell-free system as a result of localized production of 2-5A, while the transcription reaction could be completely inhibited by the addition of the 3'-O-methyl analogue of 2-5A (Baglioni *et al.*, 1984). There is no direct evidence for specific and selective cleavage of viral RNA as a response to localized 2-5A production in intact IFN-treated cells. In fact, if this were the predominant means by which the cellular machinery distinguished between viral and host RNA, the well-described cleavage of rRNA in IFN-treated EMCV-infected cells would be aberrant (Silverman *et al.*, 1983), although perhaps understandable if the latter cleavage took place only in the presence of large 2-5A concentrations.

4.5. Affinity Labelling of RNase L

Affinity labelling of RNase L has been accomplished through a variety of approaches. Crosslinking of RNase L to alkaline phosphatase-digested periodate-oxidized $[^{32}\text{P}]\text{ppp}5'(\text{A}2'p5')_n\text{ApCp}$ was accomplished by Wreschner *et al.* (1982). Simple UV irradiation was used to crosslink 2-5A to RNase L in a study by Floyd-Smith *et al.* (1982). Photoactivatable azido derivatives of 2-5A also were reported

(Suhadolnik *et al.*, 1988), but their usefulness was greatly restricted by their low specific activities. Nolan-Sorden *et al.* (1990) reported the use of a brominated 2-5A derivative, $\text{p}(\text{A}2'p)2(\text{br}^8\text{A}2'p)_2\text{A}3'[^{32}\text{P}]\text{Cp}$, as a highly effective photoaffinity label. The affinity-labelled protein migrated to a molecular mass of 85 kDa on SDS-PAGE and the affinity labelling could be prevented by competition with $\text{ppp}5'(\text{A}2'p5')_n\text{A}$, but not $\text{ppp}5'\text{A}2'p5'\text{A}$.

4.6. Activation of RNase L by 2-5A and Analogues

Examination of structure-activity relationships using 2-5A and its analogues and RNase L have generated considerable information on the nature of the 2-5A-binding domain. The driving force behind this work has been a desire to employ 2-5A derivatives in intact cells in order not only to determine the biological role of the 2-5A system, but also to evaluate its potential for antiviral and cancer chemotherapy. In order to accomplish this goal, several considerations must be addressed. Firstly, 2-5A has a short half-life in biological systems, resulting from the activity of 2',5'-phosphodiesterase, and secondly, because of the highly ionic nature of 2-5A, it does not easily penetrate the cell membrane of the intact eukaryotic cell. In order to provide information to mitigate these factors, structural components necessary for binding and activation have been closely evaluated using chemical and enzymatic methods.

To examine binding of the 2-5A analogue to RNase L, a 2-5A tetramer, e.g., $\text{ppp}5'(\text{A}2'p5')_3\text{A}$, has been linked to $[^{32}\text{P}]\text{pCp}$ using T4 RNA ligase (Knight *et al.*, 1980). The resultant radioactive probe may be competed with a number of 2-5A analogues and the binding IC_{50} measured and compared with 2-5A. Several other techniques have been used to evaluate activation of RNase L so that the relative contribution to binding versus activation of various 2-5A structural domains may be compared (Lewis and Falcoff, 1981). An early method involved the ability of the analogue to inhibit cell-free protein synthesis from a specific mRNA (Torrence and Friedman, 1979). Another method uses the property that 2-5A causes specific cleavages in rRNA that can be evaluated in a cell-free system and examined by agarose gel electrophoresis (Wreschner *et al.*, 1981a). Perhaps the most frequently employed assay has been the use of poly(rU) 3'-ligated to $[^{32}\text{P}]\text{pCp}$. The resultant poly(rU)- $[^{32}\text{P}]\text{pCp}$ product was evaluated as a substrate for RNase L by measurement of trichloroacetic acid-precipitable radioactivity in response to activation of the enzyme by a 2-5A analogue. In order to distinguish cleavage caused by RNase L, as opposed to other cellular nucleases, the RNase L first must be immobilized on core 2-5A cellulose and washed free of contaminating nucleases. In contrast, with purified human recombinant enzyme, Carroll *et al.* (1996) were able to employ $[\text{P}^{32}]\text{5}'\text{-pC}_{11}\text{UUpC}_7$ as a synthetic substrate.

Although 2-5A synthetase can generate 2-5A oligomers from dimers to products longer than decamers, oligoadenylation must be trimers or longer in order to effectively bind

to and to activate RNase L (e.g., Torrence *et al.*, 1984; Johnston and Torrence, 1984). Oligomers of greater length than the trimer bind to and activate the enzyme approximately equally well. There is limited evidence for tissue-specific differences in oligomer size activation requirements for RNase L (Krause and Silverman, 1993; Wu *et al.*, 1983). RNase L from rabbit reticulocyte lysates, thus far, is unique in requiring tetramer 2-5A instead of trimer 2-5A for activation, as measured by the core-cellulose assay and by their respective abilities to cause an inhibition of translation in a reticulocyte cell-free system. Rabbit kidney cell RNase L, however, as well as murine reticulocyte RNase L, were activated by trimer or tetramer 2-5A.

4.6.1. Dependence of RNase L activity on nature of ribose-phosphate backbone. The most intriguing feature of the 2-5A molecule is the presence of the 2',5'-phosphodiester bonds themselves. Might one or all of these bonds be replaced by 3',5'-linkages without impeding binding to and activation of RNase L? 3-5A linkage isomers were prepared either by lead ion-catalyzed polymerization of adenosine 5'-phosphorimidazolates or by means of T4 polynucleotide kinase-catalyzed 5'-phosphorylation of A3'p5'A3'p5'A, followed by reaction of the corresponding phosphorimidazolates with tri(*n*-butylammonium)pyrophosphate (Lesiak *et al.*, 1983). When ppp5'A3'p5'A3'p5'A was evaluated as a translational inhibitor using EMCV RNA-programmed L-cell extracts, it was found to be approximately 10,000 times less efficacious as 2-5A (Lesiak *et al.*, 1983). Phosphodiester linkage analogues of 2-5A, ppp5'A2'p5'A3'p5'A, and ppp5'A3'p5'A2'p5'A were 25–28 times less effective as translation inhibitors (Lesiak *et al.*, 1983). In addition, ppp5'A3'p5'A2'p5'A, ppp5'A2'p5'A3'p5'A, and ppp5'A3'p5'A3'p5'A were 48, 43, and 13,000 times less effective than 2-5A at displacing ppp5'A2'p5'A2'p5'A3'[³²P]-p5'C3'p in the radiobinding assay. Monophosphates of these linkage isomers showed a similar pattern of activity (Lesiak *et al.*, 1983). The above observations have been confirmed using purified recombinant human enzyme. It follows that 2',5'-phosphodiester bonds are stereochemical requirements for the biological activity of 2-5A.

Another structural feature that may be examined is the role of the ribose moiety. In particular, replacement of ribose by 3'-deoxyadenosine (cordycepin) might be a particularly important modification with regard to improving resistance towards nuclease degradation, since certain nucleases that may be involved in 2-5A breakdown operate through a 2',3'-cyclic phosphate intermediate, the formation of which would be blocked in such 3'-deoxyadenosine analogues.

Although the enzymatic synthesis of ppp5'(3'dA)2'p5'(3'dA)2'p5'(3'dA)2'p5'(3'dA) by 2-5A synthetase has been reported (Doetsch *et al.*, 1981), structural proof consisted solely of an R_f comparison of a radiolabelled alkaline phosphatase-digested product to the well-characterized chemically synthesized core (Charubala and Pfeleiderer,

1980a,b) in a single TLC system. Although the ppp5'(3'dA)2'p5'(3'dA)2'p5'(3'dA)2'p5'(3'dA) was found to be a more potent translational inhibitor than 2-5A trimer or tetramer in a rabbit reticulocyte cell-free system (Doetsch *et al.*, 1981), other groups (Silverman *et al.*, 1981; Williams *et al.*, 1979a) have found 2-5A trimer not to be a subnanomolar activator of the nuclease at all, in contrast to other rabbit cell types and all murine and human lines examined thus far.

The 5'-di- and -triphosphates and core of the cordycepin analogue of 2-5A trimer and tetramer have been prepared and characterized by four groups (Sawai *et al.*, 1983; Haugh *et al.*, 1983; Nyilas *et al.*, 1986; Charubala and Pfeleiderer, 1980a,b). The products were characterized both enzymatically and physically. The core oligonucleotide, produced by Charubala and Pfeleiderer, was identical after phosphorylation to the 5'-monophosphate produced by Sawai *et al.* (1983), and the alkaline phosphatase digestion product of the latter also was found to be identical with the Charubala and Pfeleiderer core trimer product. These cordycepin analogues of 2-5A have been examined in a number of different assay systems. In L_k-cell lysates, (pp)p5'(3'dA)2'p5'(3'dA)2'p5'(3'dA)2'p5'(3'dA) was found to bind to RNase L 20 times less well than 2-5A and to activate the nuclease 4 × 10⁵ less potently than 2-5A, as judged by inhibition of EMCV RNA translation (Sawai *et al.*, 1983). In a globin mRNA-programmed rabbit reticulocyte cell lysate, the cordycepin 2-5A trimer analogue was active only at micromolar concentrations or above, as was 2-5A itself; however, when the tetramers were evaluated under the same conditions, the cordycepin analogue was more than 10³-fold less potent than 2-5A (Sawai *et al.*, 1983). Haugh *et al.* (1983) did not find activation of RNase L in L-cell or Daudi cell extracts by pp5'(3'dA)2'p5'(3'dA)2'p5'(3'dA). Krause *et al.* (1986) found no activity of ppp5'(3'dA)2'p5'(3'dA)2'p5'(3'dA)2'p5'(3'dA)2'p5'(3'dA) in the core-cellulose/poly(U)pCp degradation assay. When calcium co-precipitation was used to introduce ppp5'(3'dA)2'p5'(3'dA)2'p5'(3'dA) into intact L-cells, it was found to be 100 times less potent than 2-5A, as measured by inhibition of protein synthesis (Eppstein *et al.*, 1985). When ppp5'(3'dA)2'p5'(3'dA)2'p5'(3'dA) was examined for its activity in mouse EAT cells, none was found, while activity in L-cell extracts was minimal (Nyilas *et al.*, 1986). Since replacement of the ribose moiety by cordycepin in all positions resulted in virtually complete loss of activity, the effects of introduction of one 3'-deoxyadenosine at each position of the trimer were examined (Torrence *et al.*, 1988). When ppp5'(3'dA)2'p5'A2'p5'A was evaluated, binding was equivalent to that of 2-5A and activation was approximately 3 times less potent than 2-5A, as measured by translational inhibition and the poly(U) degradation assay. With ppp5'A2'p5'A2'p5'(3'dA), an increase in binding and activation was observed. However, ppp5'A2'p5'(3'dA)2'p5'A displayed an 8-fold decrease in binding, while activation of RNase L was decreased 500- to 1000-fold. Therefore, the 3'-hydroxyl of the second (from the 3'-terminus) nucleotide residue of 2-5A is critical for binding to RNase L.

Baglioni *et al.* (1981a) investigated a series of 3'-O-methylated analogues of 2-5A. 2'-terminal substitution of 3'-O-methyladenosine for adenosine in 2-5A trimer gave a compound with enhanced ability to inhibit protein synthesis in a cell-free system, probably due to enhanced phosphodiesterase resistance. Methylation of all of the 3'-hydroxyl groups of 2-5A trimer, however, produced an inactive compound. These earlier results are completely consistent with the above studies on 3'-deoxyadenosine analogues, and reinforce the importance of the ribose moieties of 2-5A for activation of RNase L.

Replacement of the oxygen atom of each of the ribose rings of 2-5A with methylene gave an aristeromycin analogue with dramatically reduced ability to activate the RNase L (Sawai *et al.*, 1985b). Only a fraction of the loss in activity could be explained by a loss of RNase L binding ability.

Since fluorine can act as a hydrogen-bond acceptor (Murray-Rust *et al.*, 1983), series of fluorosugar analogues of 2-5A were synthesized for evaluation in binding to and activation of RNase L (Kovacs *et al.*, 1993). Replacement of the 3'-hydroxyl in the second position from the 3'-terminus resulted in significant decreases in both binding and activation of RNase L, despite the absence or presence of 3'-F substitution in the other residues. Therefore, the middle 3'-hydroxyl in 2-5A trimer may act as a hydrogen-bond acceptor while bound to RNase L (Kovacs *et al.*, 1993).

Beigelman *et al.* (1995) examined the biological properties of a phosphorodithioate analogue of 2-5A and found that it was about 10-fold less effective than 2-5A as an activator of RNase L, and that this reduction in activity was related to a corresponding decrease in binding to the RNase L.

The individual diastereomeric cores of A2'p(s)5'A2'p5'A were prepared by a modified hydroxybenzotriazole phosphotriester approach (De Vroom *et al.*, 1987). The 5'-monophosphates were then derived after phosphorylation with a 6-trifluoromethyl-1-benzotriazolyl-activated phosphomorpholidate.

The synthesis and resolution of P-thioadenylyl-(2'-5')-P-thioadenylyl-(2'-5')-P-thioadenylyl-(2'-5')-adenosine cores were first performed by Charubala *et al.* (1991). All diastereomers bound equally well to cell lysate-derived RNase L. In the core cellulose assay, all activated the enzyme except the SpSpSp isomer, which was an antagonist. The four diastereomers of P-thioadenylyl-(3'-5')-P-thioadenylyl-(3'-5')-adenosine were synthesized (Charubala *et al.*, 1991). None of these compounds bind to or activate RNase L.

The phosphorothioate analogues of 2-5A dimer and trimer cores, as well as their 5'-monophosphates, were prepared by the phosphoramidite method, and the diastereomers were separated by chromatographic means and fully characterized by physical means (Charubala and Pfeleiderer, 1992).

2',5'-Oligothioadenylate trimer cores have also been prepared via UO_2^{2+} ion-catalyzed polymerization of adenosine-5'-thiophosphoroimidazolide (Shimazu *et al.*, 1992). The oligomers contain a preponderance of linkages of Rp configuration. 2',5'-Oligothioadenylate dimer cores with

Sp stereoselectivity were attained using hydrogen phosphonate methodology, followed by oxidative phosphorylation (Battistini *et al.*, 1992).

The phosphorothioate bonds of the 2-5A trimer core in which both internucleotidic bonds were of Sp configuration were very stable to serum and cellular and serum phosphodiesterases (Eppstein *et al.*, 1986). The all Rp 2-5A core trimers, however, while more stable than their all phosphodiester 2-5A analogues, were much less stable than the SpSp isomer. The SpSp phosphorothioate isomer had a half-life of over 30 days in serum.

5'-O-[(Sp)-1-P-thiotriphosphoryl]-(Rp)-P-thioadenylyl(2'-5')adenosine, prepared by enzymatic means, was found to bind to and to activate RNase L (Kariko *et al.*, 1987a,b). This analogue displaced $\text{p}_3\text{A}_4[^{32}\text{P}]\text{pCp}$ from cell lysate-derived RNase L with an IC_{50} of 5×10^{-7} M compared with an IC_{50} of 5×10^{-9} M for p_3A_3 . Poly(U)-3'-[^{32}P]pCp is 20% degraded at 2×10^{-7} M, while p_3A_2 does not hydrolyze this substrate under the same conditions. The corresponding RpRp trimer triphosphate was also prepared, characterized, and evaluated. The four phosphorothioate trimer core and 5'-monophosphate analogues were also prepared and used to dissect the structural parameters governing binding and activation to RNase L of these stereoisomers (Kariko *et al.*, 1987a). Binding of the four diastereomeric pairs was not appreciably different; however, activation parameters, as determined by rRNA and core-cellulose assays, showed marked differences between the isomers. The order of activation of RNase L was identical for core and 5'-monophosphate analogues with $\text{RpRp} > \text{SpRp} > \text{RpSp}$. The pSpSp and SpSp analogues are ineffective at activation and function as antagonists in competition assays. When evaluated as antiviral agents following cytoplasmic microinjection into HeLa cells, the pRpRpRp, pSpRpRp and pRpSpSp diastereomeric 2-5A phosphorothioate tetramer analogues demonstrate antiviral activity versus VSV (Charachon *et al.*, 1990). The pSpSpSp analogue again acts as an antagonist.

4.6.2. Dependence of RNase L activity on the nature of the bases. The requirement for base specificity by RNase L was established when it was found that complete substitution of adenine for any other base, including cytosine, hypoxanthine, uracil, or ethenoadenosine, resulted in a loss of activity (Torrence *et al.*, 1984; Lesiak and Torrence, 1983). When the 3'-terminal base was replaced by any common nucleotide to form ppp5'A2'p5'A2'p5'N, a loss of RNase L activating ability resulted, although antagonism of the action of 2-5A was still possible (Drocourt *et al.*, 1982). Further, replacement of the first base by hypoxanthine to give ppp5'I2'p5'A2'p5'A resulted in a 200-fold loss of activating ability, as well as binding affinity, while replacement of the second base to yield ppp5'A2'p5'I2'p5'A yielded a 20-fold decrease in activating ability, but only a 3-fold decrease in binding affinity (Imai and Torrence, 1985; Imai *et al.*, 1985). Replacement of the 3'-base by hypoxanthine to give ppp5'A2'p5'A2'p5'I resulted in a 1000-fold loss of activating ability, as measured by inhibition of translation, but no

decrease in binding affinity. Therefore, the N¹/N⁶ of the 5'-terminal adenosine is required for binding to the RNase L, while the N¹/N⁶ (the 3'-terminal adenosine) was required for effective activation of RNase L, but not for binding. Further evidence for the importance of the N¹/N⁶ moiety of the 5'-terminal adenosine was shown by the synthesis of ppp5'G2'p5'(c⁷A)2'p5'(c⁷A), which was over 100 times less effective in binding to murine RNase L (Jamouille and Torrence, 1986).

Uridine substituted at various positions in the 2-5A trimer further substantiates results obtained with the inosine-modified 2-5A analogues (Kitade *et al.*, 1991a). Thus, while 5'-residue substitution to form ppp5'U2'p5'A2'p5'A caused a 100-fold decrease in both binding and activation, and while replacement of the terminal 2'-residue caused a 10⁴ decrease in activation, replacement of the middle adenosine to form ppp5'A2'p5'U2'p5'A showed a correspondingly larger decrease in activation ability than the inosine 2-5A analogues, as expected from the inosine analogue studies.

Formation of sequence-specific analogues of 2-5A with tubercidin (7-deazaadenosine, c⁷A) further corroborates the results mentioned above (Jamouille *et al.*, 1984, 1987). The tubercidin 2-5A analogue ppp5'(c⁷A)2'p5'(c⁷A)2'p5'(c⁷A) bound almost as well as 2-5A trimer, but was incapable of activation of the nuclease. When ppp5'A2'p5'A2'p5'(c⁷A) and ppp5'(c⁷A)2'p5'A2'p5'A were evaluated, a large decrease in activation ability with more moderate loss of binding ability was observed, while ppp5'A2'p5'(c⁷A)2'p5'A showed a negligible reduction of activation ability, despite a moderate loss of binding ability. Thus, the situation is somewhat reminiscent of the inosine and uridine analogues; that is, the base moiety of the second nucleotide unit of 2-5A appears to play a less important role in RNase L activation than do the first or third adenines of 2-5A trimer. It would also appear that the purine N7 of these latter two adenine sites of 2-5A may be important contributors to the binding/activation process (Jamouille *et al.*, 1987).

Substitution of bromine for the purine 8-position H also moderates binding and activating ability (Lesiak and Torrence, 1986, 1987). While (p)pp5'A2'p5'(br⁸A)2'p5'A and (p)pp5'(br⁸A)2'p5'A2'p5'A led to over a 10²-fold decrease in activation directly attributable to decreases in binding affinity, pp5'A2'p5'A2'p5'(br⁸A) activated murine RNase L 10-fold more effectively than 2-5A, as measured by inhibition of translation. Conformational studies on these brominated 2-5A analogues revealed that good binding to RNase L requires an *anti* orientation of the base about the glycosidic bond of the 5'-terminal adenosine (van den Hoogen *et al.*, 1989). In addition, a *syn* base-sugar orientation or an S-type sugar conformation of the 2'-terminal adenosine may positively influence binding to RNase L. Studies on 8-methyladenosine analogues of 2-5A further confirmed these results (Kitade *et al.*, 1991b). Introduction of the 8-methyladenosine into the 5'-terminal position resulted in a decrease in binding affinity, with a concomitant decrease in activating ability, while introduction into the 2'-terminus yielded an analogue that activated better than 2-5A itself.

RNase L derived from most lines of mouse L-cells requires a 5'-triphosphate for effective activation. However, several rational modifications of 2-5A can obviate the necessity for the polyphosphate, which in and of itself presents problems referable to increased charge and diminished cellular uptake, as well as increased susceptibility to cellular phosphatases and phosphodiesterase, both of which tend to decrease biological activity of the 2-5A. Although the 5'-diphosphate can substitute for the 5'-triphosphate in most L-cell lines (Lesiak and Torrence, 1985), this requirement is not absolute (Kovacs *et al.*, 1993), as one L-cell line and a mouse liver extract require only a 5'-monophosphate. In the former cases, the 5'-monophosphates can serve as effective antagonists (Torrence *et al.*, 1981a). Several derivatives have been found to mitigate the necessity for the 5'-polyphosphate. The p5'A2'p5'A2'p5'(br⁸A)2'p5'(br⁸A) tetramer has been found to be as effective an activator as ppp5'A2'p5'A2'p5'A in mouse L-cells, as determined by inhibition of translation, as well as being more stable toward cellular phosphodiesterases (Torrence *et al.*, 1992).

4.6.3. Other modifications. Baglioni *et al.* (1981a) reported that the β,γ -methylene phosphonate analogue of 2-5A trimer triphosphate could not activate the enzyme, as judged by protein synthesis inhibition, but still could bind to RNase L, as ascertained by its ability to inhibit the action of 2-5A itself. In contrast, a β,γ -difluoromethylene analogue showed significant activity, possibly due to a closer correspondence of the pKa's of the polyphosphate moiety to natural 2-5A, as compared with the unsubstituted methylene analogue (Bisbal *et al.*, 1987). In addition, a β,γ -phosphorothioate analogue of 2-5A and a β -thiophosphate analogue both were effective activators of RNase L (Bisbal *et al.*, 1987). A uronic acid analogue of 2-5A showed a trace of activity in RNase L activation (Kinjo *et al.*, 1992).

Several additional modifications of 2-5A are discussed below (Section 5) in relation to antagonism of RNase L and IFN action (see also Table 10).

4.7. RNase L, 2-5A, and the Antiviral Action of Interferon

Considerable evidence exists relating RNase L to the antiviral activity of IFN. In a virus-infected IFN-treated cell, dsRNA production activates 2-5A synthetase to produce 2-5A, which in turn activates RNase L. The RNase L causes a generalized depletion of viral as well as cellular RNA, resulting in impaired viral replication. Member viral species from several families, including the picornaviridae, reoviridae, and poxviridae, have been examined. On the other hand, an IFN-mediated antiviral response in the absence of detectable 2-5A synthetase or RNase L has been reported (Meurs *et al.*, 1981).

Picornaviruses are naked icosahedral nucleocapsids of 25–30 nm diameter, having a core of ssRNA. They are subdivided into four genera: enterovirus, cardiocivirus, rhinovi-

TABLE 10. Compounds that Have Demonstrated Ability to Antagonize the Action of 2-5A

Oligonucleotide	Reference
p5'A2'p5'A2'p5'A p5'A2'p5'A2'p5'A2'p5'A	Torrence <i>et al.</i> , 1981a, unpublished Miyamoto <i>et al.</i> , 1983; Haugh <i>et al.</i> , 1983; Jamouille <i>et al.</i> , 1984; Black <i>et al.</i> , 1985; Lesiak and Torrence, 1986
p5'A2'p5'A2'p5'A-N-hexylmopholine p5'A2'p5'A2'p5'A2'p5'A-N-hexylmopholine	Imai <i>et al.</i> , 1982a
ppp-, pp-, and p5'(3'dA)2'p5'(3'dA)2'p5'(3'dA) and tetrameric congeners	Sawai <i>et al.</i> , 1983
p5'A2'p5'A2'p5'A2'p p5'A3'p5'A2'p5'A p5'A2'p5'A3'p5'A p5'A3'p5'A2'p5'A2'p5'Ap5'A2'p5'A3'p5'A2'p5'A	Torrence <i>et al.</i> , 1984 Lesiak <i>et al.</i> , 1983
CH ₃ Sp5'A2'p5'A2'p5'2'3'CH ₂ CH ₃ Sp5'A2'p5'A2'p5'A2'pp3'RCH ₃	Haugh <i>et al.</i> , 1983 Watling <i>et al.</i> , 1985
β-γ-CF ₂ -A ₄ (ox-red) ppp5'- and p5'A2'p5'A2'p5'1	Bisbal <i>et al.</i> , 1987 Imai <i>et al.</i> , 1985

rus, and aphthovirus. The replicative cycle is significant in its capacity for the production of large amounts of dsRNA. For instance, with the poliovirus, a well-studied member of the enterovirus genera, the parental genome functions as mRNA. After production of the polyprotein, double-stranded replicative forms are transcribed that can result in the production of progeny plus strands by a peeling off mechanism.

EMCV, a cardiovirus that causes mild febrile illnesses in a number of species, including humans, has been often evaluated in conjunction with the 2-5A system. Accumulation of 2-5A as measured by rRNA cleavage has been observed in EMCV-treated, IFN-treated cells (Wreschner *et al.*, 1981a; Silverman *et al.*, 1982, 1983; Williams *et al.*, 1979b; Knight *et al.*, 1980), as well as the organs of EMCV-infected mice (Hearl and Johnston, 1987). The observed elevation of 2-5A provides direct evidence for stimulation of 2-5A synthetase by viral dsRNA. Poliovirus, however, has not been observed to induce rRNA cleavage products, possibly because of the short half-life of the replicative intermediates (Munoz *et al.*, 1983).

Other evidence implicates the 2-5A system in the antiviral effects of IFN. Increased levels of resistance to EMCV infection occur in human glioblastoma T98G cells stably transfected with a human 2-5A synthetase cDNA, which results in constitutive 2-5A synthetase production (Rysiecki *et al.*, 1989). In other similar studies, transfection of murine cDNAs encoding the 43-kDa 2-5A synthetase into NIH 3T3 cells results in varying levels of constitutive 2-5A synthetase production, as well as greatly increased resistance to EMCV infection, but not to VSV infection (Coccia *et al.*, 1990; Chebath *et al.*, 1987b). Transfection of a competitive inhibitor, CH₃Sp(A2'p)₂A2'pp3'OCH₃, of RNase L by calcium phosphate co-precipitation greatly reduces the anti-EMCV effects of IFN, as measured by the absence of characteristic rRNA cleavage patterns (Watling *et al.*, 1985).

A truncated murine RNase L (clone ZB1) acted as a dominant negative mutant of RNase L (Hassel *et al.*, 1993).

This mutant RNase L was truncated by 89 C-terminus amino acid residues, and while 2-5A binding activity was present, catalytic activity was completely absent. When stably expressed in murine SVT2 cells, this truncated version of RNase L caused an inhibition of the 2-5A system. Specifically, expression of the SVT2/ZB1 mutant prevented 2-5A-dependent rRNA cleavage, and the antiviral effect of IFN-α/β for EMCV was reduced by 250-fold while the anti-VSV effect was reduced only 2.5-fold. The antiproliferative effect of IFN was also suppressed, although the IFN-mediated induction of PKR was not. One further intriguing result that requires additional study is that in the absence of IFN when cells are infected with EMCV, a drastic decrease in RNase L occurs, and this decrease is prevented by the pre-treatment of the cells with IFN (Cayley *et al.*, 1982a). This clearly represents a viral defense against the 2-5A system.

Reoviruses are naked icosahedral, doubly encapsulated nucleocapsids of approximately 75 nm diameter, having a core of multiply segmented dsRNA. There are six nonantigenically related genera, the most significant for human disease being the genus rotavirus. Significant quantities of dsRNA are produced as replicative intermediates in the reovirus life cycle. Reovirus infection may cause 2-5A accumulation, as well as characteristic rRNA breakdown, and this may be partially compensated for by cells by means of an increase in mRNA synthesis (Nilsen *et al.*, 1982a, 1983).

Poxviruses are 25–300 nm complex brick-shaped particles containing complex DNA genomes. Vaccinia virus, a member of the orthopoxvirus genus, possesses a replicative cycle that involves the production of a dsRNA. A number of studies have found large or small increases in 2-5A levels, activation of RNase L, and characteristic rRNA breakdown in vaccinia-virus infected, IFN-treated cells (Rice *et al.*, 1985; Goswami and Sharma, 1984). Several vaccinia proteins may mitigate or enhance the ability of the 2-5A system to participate in the antiviral activity of IFN. For instance, mutations in the A18R gene, the expression product of which may play a role in viral replication, effectively en-

hance the levels of 2-5A synthetase and RNase L. Specific methods by which poxviruses may escape 2-5A system control remain to be determined.

5. AGENTS OF INTEREST AS ANTAGONISTS OF INTERFERON ACTION

Molecules that may inhibit or antagonize the action of 2-5A (Table 10) may be of interest for several reasons. They could block the effects of 2-5A to allow an assessment of its biochemical importance in a particular biological situation; for instance, what role does the 2-5A system play in inhibition of the growth of picornaviruses in IFN-treated cells? Similarly, by inhibiting the action of RNase L, such agents may permit the assessment of the relative importance of the 2-5A system versus another biochemical mechanism in a given situation; for example, what is the relative importance of the 2-5A system in the mechanism of inhibition of protein synthesis by dsRNA? In addition, to the extent that 2-5A or the 2-5A system might be involved in differentiation, cell growth regulation, or the pathological manifestations of IFN production and action, such antagonists (or analogue inhibitors) may lead to chemotherapeutic applications.

The first agent reported as an antagonist of 2-5A was the oligonucleotide p5'A2'p5'A2'p5'A (Torrence *et al.*, 1981a). Addition of 200 μ M p5'A2'p5'A2'p5'A to an L-cell-free protein synthesis system resulted in a 300-fold increase in the concentration of 2-5A required to inhibit protein synthesis. This blockade of 2-5A-mediated translational inhibition was paralleled by a similar prevention of the 2-5A-mediated enhancement of nuclease activity. Thus, the oligonucleotide p5'A2'p5'A2'p5'A was able to bind to the RNase L, but it was unable to activate it for degradation of RNA.

Subsequently, the role of 2-5A in the translational inhibitory effects of dsRNA in extracts of IFN-treated cells was examined using p5'A2'p5'A2'p5'A as an antagonist. In fact, in an EMCV RNA-programmed, IFN-treated, mouse L-cell-free system, p5'A2'p5'A2'p5'A was able to prevent the action of poly(I)·poly(C). At the highest concentration (200 μ M) of p5'A2'p5'A2'p5'A tested, and with poly(I)·poly(C) at 2 μ M, up to 80% relief of the dsRNA-mediated inhibition of translation could be obtained (Torrence *et al.*, 1981a). Studies conducted in the above cell-free system, but using phosphodiesterase-stabilized antagonists, such as p5'A2'p5'A2'p5'A2'p or the N-hexylmopholine-terminated 2',5'-oligoadenylate, revealed that even when allowance was made for the substantial concentrations of 2-5A generated by the poly(I)·poly(C)-activation of 2-5A synthetase, a significant translational inhibitory component, not preventable by the 2-5A antagonists, remained. Thus, while 2-5A was the primary mediator of the poly(I)·poly(C)-induced translational repression in this particular cell-free system under given conditions, another mechanism (protein kinase?) apparently was operational also (Torrence *et al.*, 1981a).

The relative contribution of the 2-5A-dependent ribonuclease to the mechanism of dsRNA action (Lengyel, 1987) can vary, depending upon the exact particulars of the system under investigation. For instance, studies of Miyamoto *et al.* (1983) confirmed the previously reported studies (Torrence *et al.*, 1981a) that the 5'-monophosphate 5'A2'p5'A2'p5'A could block the activation of RNase L by 2-5A. Specifically, it could block the 2-5A-mediated degradation of reovirus mRNA. However, in contrast to the results of Torrence *et al.* (1981a), Miyamoto *et al.* (1983) found that reovirus mRNA translational inhibition by reovirus dsRNA in extracts of IFN-treated mouse cells was only partly prevented (30–35%) by p5'A2'p5'A2'p5'A, in distinct contrast to the 80% prevention of poly(I)·poly(C) action seen earlier (Torrence *et al.*, 1981a). The remaining inhibitory activity of reovirus dsRNA was ascribed to the PKR (Miyamoto *et al.*, 1983). These differences emphasize that what would appear to be relatively minor differences in conditions may lead to major shifts in the mechanism by which dsRNA operates to inhibit protein synthesis, at least in cell-free extracts. Whether such would be the case in intact cells remains a subject of inquiry. It might be expected that with such a parallel functioning of the two dsRNA-activatable systems, it may be difficult to dissect their relative contributions to IFN action.

The antagonism of 2-5A action by p5'A2'p5'A2'p5'A in mouse L-cell-free extracts was extended to intact cells (Black *et al.*, 1985). Calcium phosphate was used to introduce the p5'A2'p5'A2'p5'A into cells. Under these conditions, p5'A2'p5'A2'p5'A (10^{-4} M) was able to prevent the protein synthesis inhibitory effects of 2-5A (10^{-6} M).

While p5'A2'p5'A2'p5'A has been an effective inhibitor of the activation of RNase L in mouse L-cell extracts and in some lines of mouse L-cells (Torrence *et al.*, 1981a; Miyamoto *et al.*, 1983; Black *et al.*, 1985; Haugh *et al.*, 1983), it does not have general applicability to other cell-free systems or other intact cells. In some other systems, such as some cultures of mouse L-cells (Haugh *et al.*, 1983) or in cell-free extracts of Daudi lymphoblastoid cells or HeLa cells (Haugh *et al.*, 1983; Lesiak and Torrence, 1987), the 5'-monophosphate mimics the action of 2-5A. In some cases, this could be due to the conversion of the 5'-monophosphate to the corresponding 5'-di- or 5'-triphosphate. In the case of human cells, however, it is now established that the pure human RNase L is activated equally well by 2-5A as 5'-triphosphate or as the 5'-monophosphate (Dong *et al.*, 1994). Thus, p5'A2'p5'A2'p5'A cannot be used as an antagonist of 2-5A action in human-derived systems.

Following the lead of Torrence *et al.* (1981a), Watling and co-workers (1985) employed an analogue-inhibitor (antagonist) of 2-5A action in intact cells. This molecule consisted of a trimeric 2',5'-oligoadenylate with a terminal 5'-monophosphate modified to a methylthiophosphate for phosphatase protection and a 2'-terminus protected with a 3'-O-methyl group to provide resistance to phosphodiesterase degradation and a 2'-pyrophosphate to enhance precipitation with calcium phosphate used in the transfection.

tion procedure to introduce the 2-5A analogue into cells. Upon calcium phosphate transfection, this analogue inhibitor (abbreviated as CH₃pA2'p5'A2'p5'A3'OCH₃2'p) could block activation of RNase L by 2-5A itself in both cell-free extracts and in intact cells. Most importantly, in IFN-treated and encephalomyocarditis-infected cells, this analogue, after calcium phosphate-aided uptake, could inhibit 2-5A-mediated rRNA cleavages, and it also could restore partially the synthesis of EMCV RNA synthesis and virus yield. The partial reversal of virus replication by CH₃pA2'p5'A2'p5'A3'OCH₃2'p was taken as evidence to implicate the 2-5A system in the inhibition of EMCV growth by IFN (Watling *et al.*, 1985). However, since the reversal was at best only 20%, it was not clear whether or not this represented the 2-5A system's maximum contribution to the anti-EMCV effects of IFN, or whether compensatory action by some different mechanism (e.g., PKR) had occurred.

6. BIOLOGICAL ROLES FOR THE 2-5A SYSTEM

A large variety of studies have implicated all or part of the 2-5A system (Fig. 2) in various biological phenomena.

6.1. Regeneration of Liver

The regenerating rat liver (Bucher and Malt, 1971) has provided a model system in which the potential role of 2-5A has been examined (Smekens-Etienne *et al.*, 1983; Etienne-Smekens *et al.*, 1983). In these studies, the levels of 2-5A synthetase, as well as 2-5A itself, were determined in both normal and regenerating rat livers. After partial hepatectomy, parallel changes in the activity of 2-5A synthetase and the concentration of 2-5A occurred. Both enzyme and oligonucleotide decreased several-fold and then increased to normal prehepatectomy activity (2-5A synthetase) and concentration (2-5A). The loss of 2-5A synthetase activity preceded the onset of labeled thymidine incorporation into DNA, synthetase activity was lowest when DNA synthesis was maximal, and when 2-5A concentrations were lowest, DNA synthesis was greatest. Thus, this system provided good correlation between 2-5A synthetase activity and/or 2-5A concentration and cell growth. These results have been interpreted as circumstantial evidence to implicate the 2-5A system in the negative control of growth, at least in the regenerating rat liver.

6.2. Estrogen Administration and Withdrawal

Another system that has provided evidence for a biological role for the 2-5A system involves estrogen administration to immature chicks. Estrogen treatment leads to differentiation and growth of the tubular gland cells in the oviduct. Egg white proteins, predominantly ovalbumin, are synthesized in such tubular gland cells (Schimke *et al.*, 1975; O'Malley *et al.*, 1977; Palmiter *et al.*, 1977). If estrogen treatment is discontinued, there results a gradual 2- to 3-week decline of ovalbumin synthesis and a decrease in oviduct size. Egg white proteins and oviduct growth may be reinduced by a variety of agents, including estrogens, progesterone, gluco-

corticoids, carcinogens, or tumor promoter (reviewed by Cohrs *et al.*, 1988), but the initial or primary stimulation occurs only with estrogens.

Rapid or acute withdrawal of estrogens, as with surgical removal of a diethylstilbestrol implant, caused inhibition of transcription of egg white protein mRNAs and a speedy degradation of existing hormone-induced mRNAs (reviewed by Cohrs *et al.*, 1988). Ovalbumin's mRNA half-life was reduced from 24 hr in the hormone-treated chick's oviduct to 2–6 hr in the hormone-withdrawn chick's oviduct. Restimulation led to new ovalbumin mRNA through transcription.

Stark *et al.* (1979) reported that 2-5A synthetase was present at very low or undetectable levels after restimulation of the oviduct with estrogen, but that during estrogen withdrawal, the activity of 2-5A synthetase rose dramatically. Cohrs *et al.* (1988) extended this observation by examining 2-5A concentrations and the fate of mRNAs in the estrogen-treated and withdrawn chick oviduct. Low concentrations (0.7 nM) of 2-5A could be detected in oviducts from chicks given secondary estrogen stimulation. Following acute estrogen withdrawal, the 2-5A concentration increased 2-fold at 6 hr post-withdrawal. 2-5A was determined using the functional nuclease assay of Silverman (1985). No HPLC evidence for 2-5A was gathered. In the further course of this study, Cohrs *et al.* (1988) found that a distinct 18S rRNA species, about 450 nucleotides in length, underwent a significant increase in intensity at 6 hr and more after acute estrogen withdrawal. This 18S rRNA-derived fragment showed an identical mobility on denaturing agarose gels as the 18S rRNA fragment produced when chick oviduct ribosomes were incubated *in vitro* with partially purified 2-5A-dependent RNase from chick oviduct. Cohrs *et al.* (1988) concluded that chick oviduct 2-5A-dependent RNase may be involved in the degradation of rRNA brought about by estrogen withdrawal and that the 2-5A-dependent RNase may play some role in the normal turnover of rRNA. Nonetheless, since no specific 2-5A-dependent RNase-derived ovalbumin mRNA product could be detected, and since the 18S rRNA-derived fragment showed no detectable increase at 3 hr after estrogen withdrawal when 35% of ovalbumin mRNA was degraded, it would seem premature to implicate the 2-5A system in the ovalbumin mRNA turnover.

6.3. Erythroid Cells

Orlic *et al.* (1981, 1984a) discovered that rat bone marrow cell cultures were inhibited in their ability to respond to erythropoietin induction of red cell formation when they were treated with 2-5A tetramer core (A2'p5'A2'p5A2'p5'A) at a concentration of 10⁻⁴ M. Not only did the 2-5A tetramer core cause a reduction of erythroid colonies, but it also inhibited [³H]thymidine incorporation. Orlic *et al.* (1982) further found that in the livers and spleens of rats made anemic by phenylhydrazine injections, there was a direct correlation between the rate of erythropoiesis and 2-5A

synthetase activity. These studies were later extended (Orlic *et al.*, 1984b, 1985) to include both hypoxia-induced and phenylhydrazine-induced erythropoiesis in mice. In the latter case, spleen cell lysates showed a 1.9- to 2.8-fold increase in 2-5A synthetase activity, as assayed by the poly(I).poly(C)-agarose method. In the hypoxia studies, 2-5A synthetase increased 102% over control values; moreover, 2-5A synthetase activity was maximal in spleen cell populations containing the largest number of nucleating cells, whereas 2-5A synthetase activity was minimal in populations carrying the fewest nucleated cells. In addition, Orlic *et al.* (1984b) found that 2-5A trimer (A2'p5'A2'p5'A) and tetramer cores partially inhibited the colony-forming response. On the basis of these foregoing results, the speculation has been advanced that 2-5A may play a role in regulating some of the late events in erythropoiesis.

Possibly relevant to the above observations is the report by Ortega *et al.* (1979) that IFN has a suppressive effect on erythroid cell proliferation. Ortega *et al.* (1979) suggested that IFN's inhibitory effect on erythropoiesis might be the cause of the acute erythroblastopenic crisis seen in certain patients with viral infections. Orlic *et al.* (1984b) then further speculated that this effect, in fact, may be mediated by the 2-5A system.

Ferbus *et al.* (1984) also examined erythropoiesis in rabbits made anemic by hemorrhage or phenylhydrazine injections. Erythrocytes formed under such conditions initially had high activity of 2-5A synthetase, but this activity decayed as the erythrocytes aged. These authors hypothesized that 2-5A synthetase, through 2-5A synthesis and resultant activation of RNase L, could be effecting the degradation of mRNA in the reticulocyte.

6.4. The Cell Cycle and 2-5A

Wells and Mallucci (1985) examined the levels and activities of the various components of the 2-5A system in highly synchronized cultures of mouse embryo fibroblasts. 2-5A synthetase activity was low in cells in G₁ and in early S phases, but increased approximately 10-fold at the peak of S phase and remained at similar high levels through the remainder of S phase. 2-5A synthetase activity dropped dramatically as the cells went through G₂ and returned to G₁. The increase in 2-5A synthetase activity was not due to its induction by autocrine IFN, since treatment of the cells with the autocrine IFN they produce brought about an increase in 2-5A synthetase from cells in the G₀ phase, but had no effect in cells that were in the G₁ or S phases of the cell cycle (Wells and Mallucci, 1988).

In the mouse embryo fibroblast system, low basal levels of 2-5A synthetase were maintained concurrent with low concentrations of a 1.7 kb 2-5A synthetase mRNA. Induction of cells in G₀ with autocrine IFN caused a considerable increase in the amounts of the 1.7 kb mRNA and a trace appearance of a 4 kb 2-5A synthetase mRNA. In contrast, the surge of S phase synthetase activity, unrelated to autocrine IFN, was related to expression of the 4 kb synthetase

mRNA (Mallucci *et al.*, 1985, 1986; Chebath *et al.*, 1986). Similar results were obtained when the IFN employed was virus-induced.

Activity attributable to a 2',5'-phosphodiesterase was higher in confluent quiescent G₁ cells, in contrast to other reports. RNase L activity rose 2-fold in S phase, also in contrast to other studies. Wells and Mallucci (1985) could not detect significant concentrations (>1 nM) of 2-5A in cell extracts by HPLC and so, concluded that if 2-5A is involved in control of the cell cycle in mouse embryo fibroblasts, it must operate at subnanomolar levels as a result of localized activation or activation for brief periods of time.

In A431 cells, IFN- γ treatment caused inhibition of growth, prolongation of the G₀-G₁ phase, and also caused dramatic morphological alterations, including an increase in cell size and production of sheets of fused cells (Kumar and Mendelsohn, 1989). In such cells, 2-5A synthetase activity was increased (approximately 3 times), as was the 1.6 kb 2-5A-synthetase mRNA. In addition, there was a differential induction and expression of the different 2-5A synthetase isoenzymes, as well as apparent differences in the enzymic activities of the three (p40, p67, and p100) isoenzymes, all of which suggested to the authors that growth inhibition of A431 cells by IFN involves the mediation of 2-5A synthetase.

In a second study, Kumar and Mendelsohn (1990) found that A431 cells treated with IFN- γ specifically synthesized increased levels of the mRNA for the polypeptide TGF- α , which is required for the growth of a variety of epithelial cells. Such IFN- γ -treated cells also secreted increased concentrations of TGF- α after 4 days of treatment. In this A431 cell system, TGF- α induced a 2-fold increase in 2-5A synthetase activity, ascribable mostly to the 100 kDa form. The authors (Kumar and Mendelsohn, 1990) suggested that these results implied regulatory interactions between TGF- α and IFN- γ , that the prolonged induction of 2-5A synthetase by IFN- γ in A431 cells could be due to the IFN- γ -induced late expression of TGF- α , that the autocrine action of TGF- α could be responsible for the high constitutive levels of 2-5A synthetase in A431 cells, and, finally, that induction of 2-5A synthetase may be a mediator of negative feedback regulation by TGF- α , as postulated for PDGF (Zullo *et al.*, 1985).

6.5. Platelet-Derived Growth Factor, Interferon, and 2-5A

PDGF is a mitogen found in platelets and is released from them during the blood clotting reaction. At the wound site, PDGF probably has many functions, including promotion of the repair of damaged tissue in cooperation with other growth factors. Treatment of cells with PDGF induces a state of "competence" (Pledger *et al.*, 1977) in such cells so that they can respond to other defined growth factors, such as insulin-like growth factor and epidermal growth factor, proceeding to engage in DNA synthesis. A variety of such "competence" genes have been described and include *c-fos*

and *c-myc* (Cochran *et al.*, 1984). Zullo *et al.* (1985) found that dsRNA was a potent inducer of the competence gene family. Conversely, the same group also reported that PDGF stimulated expression of the genes usually characterized as being dsRNA-inducible; namely, 2-5A synthetase and IFN- β . Zullo *et al.* (1985) proposed that the 2-5A synthetase and IFN genes could function in a feedback loop to control cell growth. They further speculated that the mitogenic response to PDGF may be inherently self-limited. Thus, at the same time it induces the genes necessary to trigger cell division, PDGF may also induce those genes required for the eventual control of such division.

6.6. Heat Shock

Nearly all cells, prokaryotes and eukaryotes, can respond to a sudden rise in growth temperature (heat shock) by the synthesis of a new set of proteins called hsp. Such proteins also can be elaborated upon exposure of the cells to other environmental stresses such as ethanol. Chousterman *et al.* (1987) found that hyperthermia not only induced hsp synthesis in bovine kidney cells (MDBK cells), but also induced 2-5A synthetase. An important difference in kinetics of induction was noted with the synthesis of hsp, which reached a maximum about 3 hr after the temperature shift, whereas induction of 2-5A synthetase was maximal 18 hr after the change in temperature. In addition, such heat-shocked cells released a 70 kDa factor (hsp 70) (Chelbi-Alix and Sripathi, 1994), different from IFN, which could induce 2-5A synthetase in fresh non-heat-shocked cells. Similarly to the hypothesis of Zullo *et al.* (1985), Chousterman *et al.* (1987) suggested that 2-5A synthetase might function as a feedback regulator in the cells' response to stress. According to their hypothesis, 2-5A synthetase may be part of a rescue mechanism in response to stress. This rescue would be accomplished by increasing the degradation of hsp mRNA through the action of 2-5A synthetase-generated 2-5A that would activate the 2-5A-dependent RNase.

6.7. Ethanol

Ethanol also induces 2-5A synthetase mRNA and activity, as well as hsp 70 (Chelbi-Alix and Chousterman, 1992). In contrast to the foregoing situation of heat shock itself (Chousterman *et al.*, 1987), ethanol also induces IFN synthesis and an antiviral state in MDBK cells (Chelbi-Alix and Chousterman, 1992). Both the antiviral state and the appearance of 2-5A synthetase activity could be prevented by antibody to bovine IFN- β , thereby establishing that 2-5A synthetase was induced by externalized IFN and not by hsp 70. Thus, even though the mechanisms of induction of 2-5A synthetase were different, Chelbi-Alix and Chousterman (1992) observed that cellular recovery from ethanol treatment may involve the 2-5A synthetase and that, as in the case of heat shock, 2-5A oligonucleotide itself might serve as a signal for the termination of the stress response.

6.8. Tubular Reticular Inclusions, Interferon, and 2-5A

IFN treatment can induce the formation of cytoplasmic tubular reticular inclusions (TRI), which are complexes of membranous tubules associated with the endoplasmic reticulum or perinuclear cisternae (Grimley *et al.*, 1984; Grimley and Schaff, 1976). They are typically observed in humans with acquired immunodeficiency syndrome (AIDS) or systemic lupus erythematosus, both diseases in which endogenous IFN production is increased relative to disease-free subjects. Feldman *et al.* (1988) noted that in human umbilical vein and thoracic aorta endothelial cells, IFN induced formation of TRI under conditions wherein 2-5A synthetase also was induced; however, whereas 2-5A synthetase activity remained constant at 2 days of IFN treatment, TRI continued to increase in size and number. The correlation between TRI and 2-5A synthetase induction also failed to extend to a number of other cell lines, in which no TRI formed, even though there was significant elevation of 2-5A synthetase activity.

6.9. Atherosclerotic Plaque

Smooth muscle cell proliferation is involved in the formation of human atheromatous plaque. Smooth muscle cells in such atherosclerotic lesions express major histocompatibility complex Class II antigens, and such lesions also contain T-cells that may be activated to secrete IFN- γ , which could suppress proliferation of the smooth muscle cells and act as a negative control of plaque formation. Warner *et al.* (1989) reported that immune IFN (IFN- γ) inhibited proliferation of smooth muscle cells from adult human blood vessels stimulated with the mitogens IL-1 and PDGF. IFN also delayed the transient mitogen-induced increase in *c-fos* mRNA. 2-5A synthetase activity was induced with IFN- γ , as well as other IFNs, but the time required for this response was much longer (24 hr) than the blockade seen in *c-fos* increase, implying that 2-5A system could not be completely responsible for the observed inhibition of proliferation.

6.10. Sarcolectins

Sarcolectins represent a family of proteins that bind to simple sugars, can agglutinate vertebrate cells, and can antagonize the antiviral action of IFN by accelerating the decay of the antiviral state. These proteins do not interact directly with IFN or its receptors. Jiang *et al.* (1988) found that in mouse cells, under conditions wherein IFN brings about a 4- to 8-fold increase in PKR and the 2-5A synthetase (predominantly the 100 kDa isoenzyme), the addition of sarcolectin 5 hr after IFN resulted in major decreases in both the 2-5A synthetase and PKR, as judged by enzyme activities and Western blots. Jiang *et al.* (1988) have suggested that sarcolectins may induce proteins that oppose, in an equilibrium fashion, the formation of the two IFN-induced proteins PKR and 2-5A synthetase. Since sarcolectins oppose IFN action, it is possible that they could be part of a homeostatic mechanism for the control of cell growth or

differentiation. For instance, both are found together in the human placenta during the fetal phase of pregnancy.

6.11. Growth Inhibition by Interferon

Chapekar *et al.* (1988) described a human epidermoid cell line called A431, which is highly sensitive to the growth inhibitory effects of dsRNA. Poly(I)·poly(C) did not cause secretion of IFN into the medium of cells, and anti-IFN serum did not block the growth-inhibitory effects of poly(I)·poly(C). Poly(I)·poly(C), however, did produce an induction of 2-5A synthetase activity (2- to 6-fold increase) and caused the degradation of 45S pre-rRNA, 28S and 18S RNAs. Nonetheless, no 2-5A itself could be found in poly(I)·poly(C)-treated cell extracts, a result hypothesized to be due to high 2',5'-phosphodiesterase activity (Chapekar *et al.*, 1988). The authors further speculated that the growth inhibitory effects of dsRNA in A431 cells were independent of IFN action, but rather were associated with rRNA degradation, which perhaps was caused by activation of the 2-5A system.

6.12. Endochondral Ossification

Maor *et al.* (1990) provided the first *in vivo* evidence that the 2-5A synthetase may be involved in a differentiation process; specifically, endochondral ossification. In embryonic mouse mandibular condyles, 2-5A synthetase activity was significantly lower in the proliferative zone of the condyle than in the more mature condylar zone where cartilage-specific proteins already had formed. In neonatal condyles, 2-5A synthetase activity was highest in the chondroprogenitor zone, which contains proliferating cells that have not differentiated. In condylar cartilage proper, where expression of cartilage antigens was complete, the activity of the 2-5A synthetase was decreased significantly. Also of note was that in the above system, the predominant 2-5A product in the assays to determine synthetase activity was the trimer pppA2'p5'A2'p5'A. The authors concluded that at the least, 2-5A synthetase could be used as a marker of differentiation in the developing condyle cartilage of neonatal mouse.

6.13. Myoblast Differentiation

To address whether IFN-induced enzymes may be involved in differentiation (Salzberg *et al.*, 1990a), Birnbaum *et al.* (1993) worked with primary myoblasts from embryonic tissue. In culture, these cells underwent fusion to multinucleated myotubes that showed spontaneous contraction and expressed antigens specific to muscle. Infection of rat skeletal muscle cell cultures with Moloney MSV blocked myotube formation, as well as the muscle-specific proteins myosin light chain-2, creatine kinase, and acetylcholine receptors. Viral genes were expressed in the infected cells. In contrast to uninfected cell cultures in which the growth-associated gene *c-myc* transcripts decreased over time, in the Moloney MSV-infected cultures, the *c-myc* RNA transcripts increased over time. Examination of control cultures showed

that 2-5A synthetase activity increased up to 3 days after seeding of the cells and then decreased; however, in MSV-infected cultures, 2-5A synthetase activity was reduced immediately after infection, but showed a substantial increase between days 5 and 7 in culture (3–5 days after infection). A nontransforming virus, Moloney murine leukemia virus, showed the same kinetics of 2-5A synthetase activity as did control cultures. Thus, the normal transient expression of 2-5A synthetase activity associated with muscle cell differentiation in culture is significantly altered in cell cultures infected with MSV, and this finding suggested to the authors a potential role for 2-5A synthetase in myogenesis. Birnbaum *et al.* (1993) went on to suggest that since MSV-transformed cells have an unusually high activity of 2-5A synthetase, it may be possible that the enzyme functions as a regulator of cell growth and no longer may be controlled effectively in the transformed cell.

6.14. Neuron Differentiation

A model system used to attempt to understand the differentiation of neurons is the treatment of the rat pheochromocytoma cell line PC12 with NGF. Saarma and co-workers (1986) found a 3-fold increase in 2-5A synthetase activity when PC12 cells were treated with NGF, and simultaneously, they detected a 2- to 4-fold decrease in the activity of the 2',5'-phosphodiesterase activity. In addition, 2',5'-oligoadenylates underwent an approximately 10-fold increase in the first hours after NGF treatment. The authors suggested that these changes in the constituents of the 2-5A system were associated with NGF-dependent differentiation in PC12 cells.

6.15. Growth Control Re-Establishment

A phenotypically revertant cell line of Ha-*ras*-transformed NIH 3T3 cells can be obtained after prolonged treatment with IFN- α/β . These cells maintained the revertant nontumorigenic phenotype, resisted transformation by specific oncogenes, and still expressed Ha-*ras*-specific mRNA and its protein product p21, even after being passaged in the absence of IFN (Samid *et al.*, 1987). Rimoldi *et al.* (1990) found that such revertant cells expressed elevated levels of 2-5A synthetase activity and the corresponding 1.7 kb mRNA, and that these levels were considerably higher than in normal parental cells of Ha-*ras*-transformed NIH 3T3 cells. These revertant cells showed a functional result of this enhanced expression of 2-5A synthetase in that they were more resistant to infection by mengovirus than were either normal or transformed NIH 3T3 cells. No endogenous production of IFN or mRNA for IFN- β could be detected in these revertant cells, demonstrating that these cells constitutively produce functional 2-5A synthetase in the absence of endogenous IFN production. The authors concluded that these results suggested a relationship between 2-5A synthetase expression and the re-establishment of growth control in these cells possessing the revertant non-tumorigenic phenotype.

6.16. Poly(I)·Poly(C)-Resistant 2-5A Synthetase in Autoimmune Rats

Peripheral blood lymphocytes (PBL) were examined for the presence of 2-5A synthetase in a study reported by Bonnevie-Nielsen *et al.* (1991b). PBL homogenates from Sprague-Dawley rats and from BB (autoimmune) rats were observed to have high basal levels of 2-5A synthetase activity in the absence of poly(I)·poly(C); moreover, addition of poly(I)·poly(C) at 200 mg/mL was needed to cause further activation of the synthetase, and the 2-5A synthetase activity from BB rat PBL was totally unaffected by addition of poly(I)·poly(C). PBL extracts from dog, pig, sheep, and humans were all comparable in virtual lack of 2-5A synthetase activity in the absence of dsRNA and showed increased activity as the concentration of poly(I)·poly(C) was increased. The additional remarkable aspect of this study was the finding that PBL extracts of BB rats gave rise to only putative 2-5A dimer, and this did not change by addition of increasing amounts of dsRNA. Equally unusual was the observation that PBL extracts of sheep and humans produced exclusively, or nearly so, octamer 2-5A in response to poly(I)·poly(C) stimulation. Human PBL extracts sometimes were able to produce tetramer also. Trimer 2-5A was observed only in poly(I)·poly(C)-treated extracts from human PBL pretreated with IFN. The authors suggested that the aberrant synthesis of inactive 2-5A dimers and the lack of stimulation of the 2-5A synthetase by poly(I)·poly(C) could imply a connection between the 2-5A system and the development of autoimmune insulin-dependent diabetes mellitus (IDDM) in the BB rat (Bonnevie-Nielsen *et al.*, 1991b).

6.17. 2-5A System and Transmissible Gastroenteritis Virus

Bosworth *et al.* (1989) investigated the induction of 2-5A synthetase and its potential role in the course of a coronavirus infection by transmissible gastroenteritis virus. 2-5A synthetase activity was increased 25-fold and replication of transmissible gastroenteritis virus was inhibited in cultured swine testicular cells treated with recombinant bovine IFN. In addition, in the same cells treated with IFN and infected with virus, detectable 2-5A (3.6 nM) was present. Infection of neonatal pigs with transmissible gastroenteritis virus caused IFN production and a 25-fold increase in 2-5A synthetase activity in enterocytes; however, no 2-5A oligonucleotides could be found under these *in vivo* conditions, and this was attributed to substantial dilution of the virus-infected cells with uninfected (crypt) cells. Thus, even though IFN limited the increase in virus titer in cultured cells and induced 2-5A synthetase, it could not prevent the cytopathic effect of the virus. A similar situation was obtained *in vivo* since 2-5A synthetase increases could be detected in the intestines of newborn pigs in response to IFN induction by virus and before virus-induced histology was present, yet cellular destruction proceeded. Since based on the above results dsRNA must have been generated (in sufficient amounts to activate the 2-5A synthetase), the

dsRNA-activated PKR pathway must also be ineffectual in this coronavirus infection. Establishment of the presence of functional RNase L in this system would be of interest.

6.18. Melanoma Cell Growth and Interferon

Creasey *et al.* (1983) presented evidence to implicate IFN and 2-5A synthetase in the cytostatic action of IFN and in the growth regulation of melanoma cells in culture. High-density cell cultures of nutrient-starved HS294T human melanoma cells produced low levels of IFN. Antibodies against human IFN- β made such cultures enter the S phase of the cell cycle earlier than did IFN-untreated cells when the cultures were stimulated with serum. Further analysis showed that there was an inverse correlation between 2-5A synthetase activity levels and the percentage of cells in S phase of the cell cycle in IFN-untreated melanoma cultures. Treatment with IFN caused the correlation to break down since 2-5A synthetase levels were elevated in all phases of the cell cycle. As a result of these observations, the authors postulated a correlation between the growth inhibitory property of IFN and the activity of 2-5A synthetase, with the caveat that additional direct evidence would have to be provided to give this hypothesis support.

6.19. The 2-5A System and Human Immunodeficiency Virus

A great deal of interest centers around the activity of the 2-5A system in HIV infection (Wu *et al.*, 1990). Immunologic dysfunction in HIV may include aberrancies in the production of cytokines such as IFN- α or IFN- γ . For instance, a decrease in the level of IFN- α in cultured PBMCs of patients with HIV-1 has been reported (Voth *et al.*, 1990), while in patients with AIDS, IFN- γ levels may be increased (Rossel *et al.*, 1989). There is also evidence that RNase L activity in lymphocytes of AIDS patients is absent despite high levels of 2-5A (Carter *et al.*, 1987). The 2-5A metabolic enzymes are present in the nuclear matrix (see Section 4.1), which also harbors HIV mRNA precursors (Verheijen *et al.*, 1988).

The HIV-1 gene product tat or transactivating protein causes a dramatic increase in the steady-state levels of HIV-1 long terminal repeat (LTR)-derived mRNAs. The interaction of tat with a region in the HIV LTR called TAR mediates this up-regulation of HIV mRNAs. The TAR sequence also is present in the 5' leader untranslated region of HIV mRNA as a large inverted repeat that forms a stable stem-loop structure. When the TAR element is fused to a heterologous RNA, the result is an inhibition of that RNA's translation.

Ederly *et al.* (1989), SenGupta and Silverman (1989), and Maitra *et al.* (1994) have found that the above translational inhibition is due in part to activation of the IFN-induced PKR by HIV-1 leader RNA containing the dsRNA stem-loop structure. In addition, 2-5A synthetase also is activated by purified TAR RNA (SenGupta and Silverman, 1989; Maitra *et al.*, 1994). Although, it was postulated that the activation of IFN-induced enzymes could play a role in

the inhibition of HIV, Roy and co-workers (1990) reported that HIV-1 infection of CEM cells caused a down-regulation of the p68 dsRNA protein kinase. Furthermore, stable expression of *tat* in IFN-treated HeLa cells caused a reduction in the amount of dsRNA protein kinase compared with IFN-treated control cells. The expression of 2-5A synthetase mRNA was not affected under these conditions. Thus, HIV infection would act to mitigate against potential translational inhibitory effects of TAR RNA through any mechanism involving the dsRNA protein kinase (Roy *et al.*, 1990).

Schroder and co-workers (1988, 1989) reported that the nuclear matrix of HIV-infected, as well as uninfected, H9 (human T-cells) contained 2-5A synthetase that was increased by a factor of 7.7 in the HIV-infected cells. This latter increase was accompanied by a 5- to 10-fold increase in 2-5A oligonucleotides in nuclei from HIV-1-infected H9 cells. Also increased in HIV-infected cells was an exonuclease activity that degraded 2-5A. The 2-5A-dependent RNase activity also underwent an increase to coincide with the time of maximum 2-5A synthetase activity. The RNase was also associated with the nuclear matrix, as determined by photochemical cross-linking and probably was responsible for degradation of HIV transcripts. Failure of infected H9 cells to release HIV was correlated with the presence of high concentrations of 2-5A and high levels of the 2-5A-dependent RNase. When 2-5A concentration decreased, the cells began to release HIV. Treatment of cells with 3'-azido-3'-deoxythymidine extended the duration of time during which HIV transcript degradation occurred. Schroder *et al.* (1989) suggested that a useful approach to screen for potential chemotherapeutic agents for HIV would be to search for compounds that would act to stabilize the concentration of 2-5A in order to extend degradation of HIV transcripts.

Schroder *et al.* (1990b) described the introduction of an expression vector containing the 2-5A synthetase coding region downstream of a 3'-LTR of HIV-1 into a cell line permissive for HIV infection. Expression of the vector DNA was activated *in trans* by the HIV *tat* protein. Apparently through the *tat*-mediated *trans*-activation of the 2-5A synthetase and resultant increase in 2-5A oligonucleotides, the replication of HIV was inhibited in cells transfected with the above vector. The authors Schroder *et al.* (1990b) postulated that HIV-*trans*-activated 2-5A synthetase could block HIV replication and that a possible therapeutic approach might be to embody this approach in gene therapy.

The *tat* protein is known to bind to a three nucleotide bulge in the stem-loop structure of TAR. Schroder *et al.* (1990c) demonstrated that while the TAR RNA sequence can activate 2-5A synthetase, *tat* protein can antagonize activation of the 2-5A synthetase by replacing the synthetase from the TAR RNA. Thus, *tat* can block 2-5A synthesis. So, just as with the down-regulation of the dsRNA protein kinase by HIV infection, the virus has a potential mechanism to subvert the action of the 2-5A system and its antiviral action.

Levels of 2-5A in cells decline as HIV production increases. When human T-cells were first infected with HIV, activation of nuclear matrix-associated 2-5A synthetase resulted in large quantities of 2-5A being produced, and this occurs in parallel with an increase in the activity of nuclear matrix-associated RNase L (Schroder *et al.*, 1988). Maximal 2-5A synthetase and RNase L activities are reached at 2–3 days post-infection, during which time no progeny virions are produced. This increase of 2-5A synthetase activity at 2–3 days post-infection was found to be paralleled by an increase in 2-5A synthetase mRNA (Ushijima *et al.*, 1993). This would indicate that after infection, either an induction of 2-5A synthetase gene expression or, less likely, an increase in 2-5A synthetase mRNA half-life occurs. Later in infection, 2-5A synthetase and RNase L activity decrease concomitant with the appearance of *tat* transcripts followed by an increase in viral protein synthesis and release of progeny virions. An increase in cytosolic 2-5A synthetase activity in HIV-infected human monocytes has also been reported (Baca *et al.*, 1994). It has been noted that the release of HIV can be delayed or prevented by agents that maintain the high intracellular levels of 2-5A by stimulating the dsRNA-dependent antiviral response of infected cells; for example, IFN (Ho *et al.*, 1985), lectins (Schroder *et al.*, 1990a), or mismatched dsRNA (Ampligen) (Carter *et al.*, 1987).

The *trans*-activator protein (*tat*) enhances LTR-directed gene expression by binding to the TAR (Rosen, 1991) that is present at the 5'-end of the HIV LTR, as well as all HIV transcripts. TAR has a sufficient secondary structure to bind to and to activate 2-5A synthetase *in vitro* (Schroder *et al.*, 1990c; SenGupta and Silverman, 1989). However, binding of 2-5A synthetase to TAR *in vitro* can be prevented by addition of *tat* (Muller *et al.*, 1990). Preferential cleavage of HIV mRNA may occur through localized activation of 2-5A synthetase in areas in which HIV transcripts are present in high concentration, resulting in the low levels of HIV mRNAs present during the early stage of infection. Therefore, treatments that prevent *tat* to TAR binding, such as the complexation of metal ions required for *tat* dimerization, may restore the antiviral state by enabling TAR to become capable of activating 2-5A synthetase. Thus, productive strategies for treating HIV infection using the 2-5A system may include the development of dsRNA analogues that increase 2-5A production, and nuclease-stable, phosphatase-stable, more potent 2-5A analogues (Sohol *et al.*, 1995).

6.20. Pathogenesis of Type I Diabetes and the 2-5A System

Since potential associations between development of the autoimmune disease Type I diabetes and virus infection have been reported (Rayfield, 1990; Gamble, 1980; Yoon *et al.*, 1979; Ginsberg-Fellner *et al.*, 1985), and since the fact that IFN- α has been found in insulin-secreting pancreatic β -cells (Foulis *et al.*, 1987) suggests the possible expression

of IFN-induced enzymes, Bonnevie-Nielsen *et al.* (1991a) explored whether or not the pathogenesis of Type I diabetes might be related to deficient expression or activation of the 2-5A synthetase. Both in RIN cells (derived from a rat insulinoma) and in pancreatic islets of Langerhans, rat IFN- α induced 10- to 15-fold increases in 2-5A synthetase activity in parallel with stimulation of major histocompatibility complex Class I gene expression (Bonnevie-Nielsen *et al.*, 1991a). In addition, the authors noted that 2-5A dimers were the principal product in IFN-untreated cell extracts incubated with poly(I)·poly(C), whereas the additional production of trimeric and tetrameric 2-5A required that the extracts be derived from IFN-treated cells. The authors concluded that the 2-5A synthetase was expressed and functional in insulin-producing cells, and suggested that the effect of virus infection on IFN-induced enzymes in such insulin-producing cells might be explored.

6.21. Splicing and the 2-5A Synthetase

Splicing of pre-mRNA in the spliceosome involves first a covalent linkage of the 5'-terminal guanosine of the intron by a 2',5'-phosphodiester bond to a conserved adenosine toward the 3'-terminus of the intron to form a lariat structure with the intron and the 3'-terminal exon, and then an excision of the intron as a lariat as the two exons are joined. Sperling *et al.* (1991) examined the possibility that the 2-5A synthetase may be involved in the initial generation of 2',5'-phosphodiester bonds in the first step of splicing.

Sperling and co-workers (1991) provided evidence that (1) 2-5A synthetase is constitutively expressed in HeLa cell nuclei as the 40 kDa and 100 kDa isoenzymes; (2) 2-5A synthetase activity could be partly immunoprecipitated by antibodies to components of small nuclear ribonucleoproteins; (3) 2-5A synthetase activity was present in the spliceosome (100 kDa isoenzyme); (4) antibodies to the 2-5A synthetase could inhibit *in vitro* splicing; and (5) when nuclear extracts were immunodepleted of 2-5A synthetase, they became deficient in their ability to catalyze splicing. Sperling *et al.* (1991) argued that the above observations, together with the fact that GTP can replace ATP in the elongation of a 2',5'-oligoadenylate primer by the 2-5A synthetase, imply that 2-5A synthetase indeed may be responsible for the initial step of the splicing reaction.

6.22. Trisomy 21 and 2-5A

Trisomy 21 cells from patients with Down's syndrome have elevated levels of gene products associated with chromosome 21, such as IFN- α and IFN- β receptors. Gerdes *et al.* (1993) demonstrated a direct relation between the number of IFN- α and - β receptors and the basal activity of 2-5A synthetase, and the sensitivity of this enzyme's elevation in response to IFN treatment. Specifically, trisomy 21 cells showed basal levels of 2-5A synthetase 1.57-fold above normal controls, but showed only a 187% increase in response to IFN as compared with a 251% increase in 2-5A syn-

thetase in IFN-treated controls. Gerdes *et al.* (1993) suggested that the observed increased basal levels of 2-5A synthetase could cause an inhibition of protein synthesis in trisomy-21 cells, thereby leading to reduced cell growth; furthermore, the reduced sensitivity to IFN would make the cells more susceptible to virus infection.

6.23. Yellow Fever Vaccination, Insulin-Dependent Diabetes, and 2-5A

Bonnevie-Nielsen *et al.* (1989) followed the induction of 2-5A synthetase activity after vaccination against yellow fever in control subjects and in subjects with IDDM. As measured by synthesis from [³²P]labelled ATP in extracts of PBL, 2-5A synthetase activity increases were significantly lower in subjects with IDDM as compared with matched controls after immunization with yellow fever vaccine; however, the IDDM subjects had higher basal 2-5A synthetase activities than controls, and this was held responsible for the attenuated response to vaccination. Bonnevie-Nielsen *et al.* (1989) proposed that a persistent viral infection or the presence of a foreign nucleic acid could explain the elevated 2-5A synthetase activity levels seen in the IDDM subjects' lymphocytes.

6.24. Pregnancy, Trophoblast Proteins, and the 2-5A System

In mammals, the corpus luteum undergoes regression to permit a new ovarian cycle. During pregnancy, however, the corpus luteum life span is extended so it can produce progesterone, which provides an environment conducive to conceptus development. Prevention of luteal regression depends on biochemical signals originating from the conceptus as part of maternal recognition of pregnancy. In primates, the primary conceptus luteotrophic signal is chorionic gonadotrophin.

In many ovine, porcine, and caprine species, prostaglandin F₂ α is responsible for inducing regression of the corpus luteum. During pregnancy, prostaglandin F₂ α continues to be released, but the mode of release changes from pulsatile, which is held responsible for regression of the corpus luteum. Protein factors identified as ovine trophoblastic protein (oTP-1) and bovine trophoblastic protein (bTP-1) have been found to be primarily responsible for this alteration of prostaglandin F₂ α secretion pattern (Barros *et al.*, 1991). Moreover, both oTP-1 and bTP-1 have been characterized as IFNs, being closely related to IFN- α and IFN- ω (Roberts, 1991). Similar proteins have been described for humans also (Aboagye-Mathiesen *et al.*, 1993).

Both oTP-1 and bTP-1 possess antiviral activities (Gill, 1991), and therefore, not unexpectedly oTP-1 infusion caused enhancement of 2-5A synthetase activity in the endometrium of ewes during early pregnancy. The conceptus protein bTP-1 induced 2-5A synthetase activity in Madin-Darby kidney cells as did IFN- α , - β , and - γ (Short *et al.*, 1991). In addition, both oTP-1 and bIFN- α induced 2-5A

synthetase activity in monolayers of endometrial epithelial and stromal cells (Mirando *et al.*, 1991). It has also been shown that the presence of the bovine conceptus during the period of maternal recognition of pregnancy causes induction of 2-5A synthetase activity, most probably through conceptus secretion of bTP-1. These and related findings have led to the hypothesis that the 2-5A system, after induction of the 2-5A synthetase by bTP-1, may act to alter prostaglandin synthesis or release by uterine cells and that the 2-5A system may play a vital biological role in the maintenance of early pregnancy.

How the 2-5A system might specifically function in the above mechanisms is not at all clear, especially since a mechanism for selective action of the RNase L has not become apparent yet. Clues already may be extant in the literature, however. An intriguing study by Hansen *et al.* (1991) documented the combined decreased transcription rate and increased mRNA turnover for oTP-1. The mRNA of oTP-1 has 3'-noncoding sequence motifs rich in adenosine and uridine. Could such motifs be targeted by the RNase L?

6.25. A Role for the 2-5A System in Apoptosis?

Castelli *et al.* (1997) stably transfected NIH 3T3 cells with the human RNase L gene placed under the control of an isopropylthiogalactoside-inducible *lac* promoter to give 3T3/RNaseLS cells. First, these cells expressed human RNase L to several-fold higher levels after treatment with isopropylthiogalactoside and after calcium phosphate-mediated introduction of 2-5A gave characteristic cleavage patterns of 28S rRNA. 3T3/RNaseLS cells grew significantly more slowly than their nontransfected parent counterparts due to an increase in cell death rate that was due to a 6-fold increase in apoptosis. Thus, increased RNase L expression led to an increased rate of apoptosis.

Second, mouse L929 cells undergo a large increase in apoptosis after exposure to both IFN and dsRNA. When these cells were infected transiently with a truncated version of RNase L missing the 89 C-terminal amino acids required for catalytic activity, but not for 2-5A binding, they became resistant to apoptosis following IFN/dsRNA treatment.

Third, the calcium phosphate-mediated introduction of 2-5A into L929 cells directly triggered apoptosis. In contrast, apoptosis was not triggered by mock infection nor by transfection with a closely related 2-5A analogue ppp5'A2'p5'A2'p5'U that binds to, but cannot activate, RNase L.

Fourth, human and mouse cell lines deficient in RNase L were resistant to induction of apoptosis by staurosporine, a good apoptosis inducer in the parent cell lines.

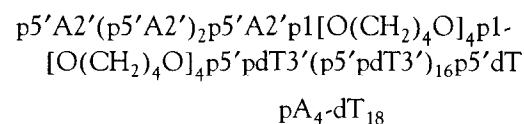
Thus, over-expression of RNase L and 2-5A activation of RNase L could induce apoptosis, whereas inhibition of RNase L action or expression prevented apoptosis.

6.26. Learning from Nature: Adapting the 2-5A System to Targeted RNA Destruction and Human Therapeutics

The 2-5A system has been the basis of a targeted mRNA destruction method that derives from the covalent linkage

of a 3',5'-antisense oligodeoxyribonucleotide and a 2',5'-oligoadenylate activator of RNase L, the 2-5A-dependent RNase (Torrence *et al.*, 1993; Lesiak *et al.*, 1993). This composite nucleic acid (Xiao *et al.*, 1996), through the antisense domain, could target the chimera to a particular mRNA sequence that would then be targeted for destruction by the 2-5A component that would provide a localized activation of the latent 2-5A-dependent RNase (Fig. 3). Presumably, this kind of chimeric molecule also could possess the mechanisms of action of classical antisense reagents, i.e., passive hybridization arrest and possibly RNase H degradation. This approach would bring into play the potent and catalytic action of the latent 2-5A-dependent endonuclease, perhaps thereby substantially increasing the potency of the antisense approach.

The prototype 2-5A/antisense chimera consisted of an antisense domain made up of oligo(dT)₁₈ connected to 2-5A through a linker (Torrence *et al.*, 1993; Lesiak *et al.*, 1993). The 2-5A and antisense moieties were joined by two 1,4-butanediol molecules joined to each other and to 2-5A and antisense by phosphodiester bonds. Linkage to the 2-5A tetramer was at the 2'-terminal hydroxyl and linkage to the antisense oligonucleotide was at the 5'-terminal hydroxyl. It was considered necessary that the mode of linkage to the 2-5A component should be through the 2'-terminus of the oligomer since a free 5'-monophosphate was requisite for maximal 2-5A-dependent endonuclease activity (Johnston and Torrence, 1984). Linker elements were used to join 2-5A and antisense rather than directly joining the terminal 2-5A adenosine nucleotide to the first nucleotide of the antisense sequence because of the possibility that the RNase L, once bound to the 2-5A component of the chimera, might disturb hybridization to target RNA, or conversely that the double helix generated from union of antisense oligonucleotide with sense RNA might interfere with maximum binding to RNase L. The structure of the chimeric oligonucleotide was:



Chimeric 2-5A-antisense was undiminished in ability to bind to RNase L. In addition, the composite molecule did not adversely affect the ability of the antisense domain to hybridize to its target poly(A), and when the poly(A) target was pre-annealed with the chimera pA₄-dT₁₈, there was no significant decrease in 2-5A-dependent RNase binding ability (Lesiak *et al.*, 1993). Thus, this novel conjugate molecule retained the ability of each partner in the construct to interact with the relevant biological receptors in a manner consistent with the proposed mechanism of action.

The 2-5A-antisense strategy for site-directed RNA cleavage first was evaluated (Torrence *et al.*, 1993) in a cell-free system consisting of a postribosomal supernatant fraction of human lymphoblastoid Daudi cells, already known to contain basal levels of the 2-5A-dependent RNase. The

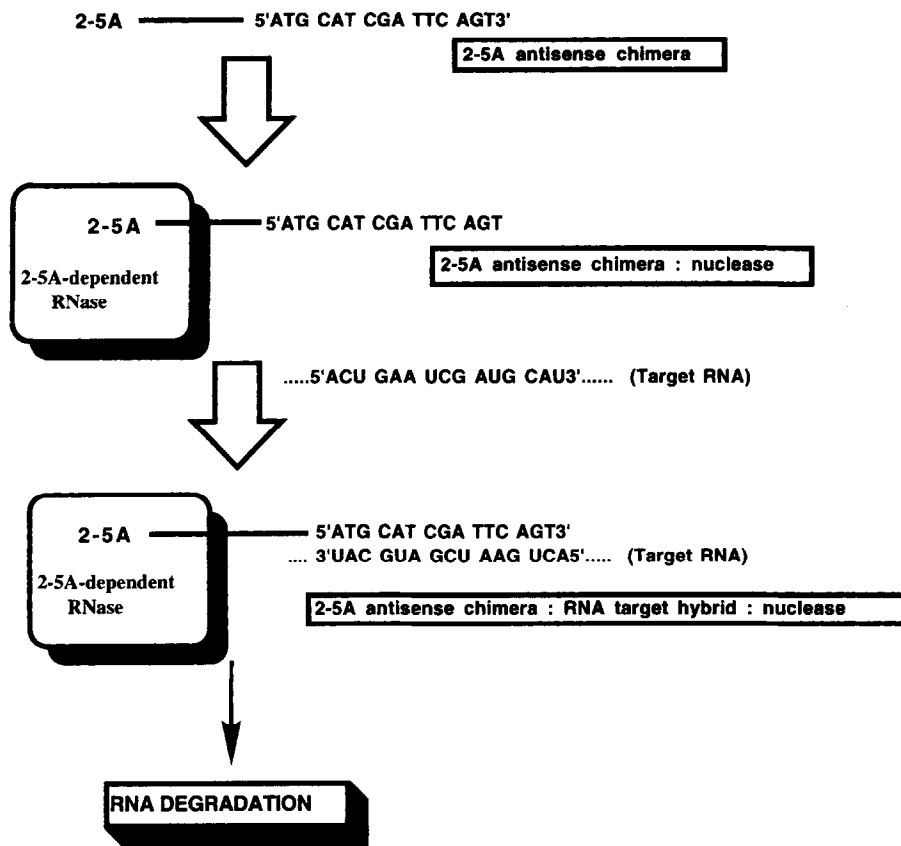


FIGURE 3. Postulated mechanism of action of 2-5A-antisense.

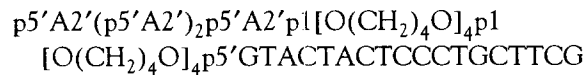
target for the pA₄-dT₁₈ chimera was an engineered modified HIV-1 *vif* mRNA containing an internal 3',5'-oligoA tract; namely, TAR:A₂₅:*vif*RNA. The resultant RNA was end-labeled with [³²P]pCp.

When the chimera pA₄-dT₁₈ was added to the cell-free system containing labeled TAR:A₂₅:*vif*RNA, there was a nearly quantitative conversion of the target RNA to a specific cleavage product. The exact sites of RNA cleavage were determined using primer-extension DNA synthesis. The chimera pA₄-dT₁₈ induced multiple cleavages within the oligo(A) tract of the TAR:A₂₅:*vif* RNA. Various control experiments provided strong evidence that cleavage of the TAR:A₂₅:*vif*RNA occurred by the postulated mechanism involving the 2-5A-dependent RNase. When 2-5A itself was added alone with unlinked (dT)₂₀, no specific cleavage of the target RNA was observed. In addition, RNA cleavage was dependent on a functionally active 2-5A derivative. Activation of the human 2-5A-dependent RNase requires a 2',5'-oligoadenylate with at least one 5'-phosphate group. Thus, in line with this requirement, the 5'-unphosphorylated compound A₄-dT₁₈ did not lead to detectable RNA cleavage. The oligonucleotide (dT)₂₀ should be able to block competitively the action of the chimera pA₄:dT₂₀ by annealing to the target A₂₅ sequence in the RNA, thus preventing access of the chimera to the RNA. Indeed, when a 10-fold molar excess of unlinked (dT)₂₀ was added to reaction mixtures containing chimeric

pA₄:dT₁₈, specific cleavage was completely blocked. The chimera pA₄:dT₄, which contains an antisense sequence too short to provide hybridization to the target A₂₅ sequence under the cleavage conditions, failed to cause specific degradation of the modified HIV RNA. If pA₄ were linked to antisense oligonucleotides that could not anneal to the A₂₅ target because they contained noncomplementary sequences, no specific cleavage resulted. The composite oligonucleotide pA₄-dT₁₈ was not capable of inducing any specific cleavages in a target RNA that lacked the A₂₅ tract; namely, TAR:*vif*RNA. Convincing proof for the vital role of the 2-5A-dependent RNase in obtaining specific cleavage of TAR:A₂₅:*vif*RNA was obtained through the use of the 2-5A analogue inhibitor of the 2-5A-dependent RNase ppp5'12'p5'A2'p5'A, an inosine-substituted congener of 2-5A. This analogue completely prevented the ability of chimeric pA₄-dT₁₈ to effect specific cleavage of TAR:A₂₅:*vif*RNA, thereby establishing the 2-5A-dependent RNase as the enzyme responsible for RNA destruction.

2-5A-antisense technology has been extended to a natural mRNA and demonstration of its specific destruction in intact cells. The target mRNA chosen codes for the protein kinase PKR, which is an IFN-inducible protein that regulates protein synthesis and can function as a tumor suppressor. The PKR mRNA site chosen as the target of the 2-5A-antisense chimera was 55–73 nucleotides from the start codon of the PKR mRNA. On this basis, we synthesized the

following 2-5A:antisense chimeric molecule directed against PKR mRNA (Maran *et al.*, 1994):



This chimera will be referred to as pA₄-antiPKR. The 2',5'-oligoadenylate moiety and the 3',5'-oligodeoxyribonucleotide antisense sequence were linked through phosphodiester bonds to two 1,4-butanediol units that in turn were joined by a phosphodiester bond.

The chimera pA₄-antiPKR was able to effect the cleavage of 5'-[³²P]labelled PKR mRNA in a cell-free system containing purified human recombinant 2-5A-dependent RNase (Maitra *et al.*, 1994). Observable cleavage was detectable at 10–20 nM of pA₄-antiPKR, with nearly complete cleavage occurring at 25–50 nM. Under the same conditions, about 400 nM of the sense orientation chimera pA₄-sensePKR was required to cause 50% cleavage of PKR mRNA (Maitra *et al.*, 1995). Thus, in a cell-free system, the composite molecule pA₄-antiPKR was a potent and selective inducer of target PKR mRNA destruction.

The chimera pA₄-antiPKR was added at 2 μM concentration to HeLa cell cultures and the cultures were incubated at 37°C for 4 hr. Total RNA was isolated from the cells, and PKR mRNA was detected using reverse transcription-coupled polymerase chain reaction methodology (RT-PCR). No signal corresponding to PKR mRNA could be detected from the pA₄-antiPKR-treated cell in contrast to the untreated cells where the PKR mRNA was clearly detectable. Thus, the chimera pA₄-antiPKR caused the complete ablation of PKR mRNA in HeLa cells. In a conclusive functional assay, it was demonstrated that cells depleted of PKR mRNA and the PKR activity were unresponsive to activation of nuclear factor-κB by the dsRNA poly(I)·poly(C), thereby providing direct evidence that PKR is a transducer for the dsRNA signaling of nuclear factor-κB. Thus, the ablation of PKR mRNA had a specific functional consequence (Maran *et al.*, 1994).

Recently, respiratory syncytial virus (RSV) replication has been targeted using 2-5A-antisense (Cirino *et al.*, 1997). Potential 2-5A-antisense oligonucleotide binding sites were selected based on computer-assisted secondary structure analysis of virus M2 mRNA. The M2 mRNA contains two ORFs, one (ORF1, nucleotides 7606–8188) for a 22 kDa internal virion protein and another (ORF2, nucleotides 8159–8431) for a protein product not yet detected in infected cells.

Appropriate 2-5A-antisense chimeras were prepared against specific sites in the computer-generated structures using second generation 2-5A-antisense chemistries. Specifically, the 5'-phosphate of the 2-5A domain of the chimera was modified to a 5'-thiophosphate that provided resistance to phosphatase action. The 3'-terminus of the antisense domain was altered to include an terminal inverted 3'-3' phosphodiester linkage that considerably increased resistance to degradation by exonucleases.

The most potent of these 2-5A-antisense chimeras spA4-antiRSV3'-3'T(8281) was directed against nucleotides 8281–8299 in the ORF2 region of the M2 RNA. For instance, in 9HTE cells, virus replication could be inhibited approximately 50% by twice daily treatment with 1 μM spA4-antiRSV3'-3'T (8281). In MA-104 cells, spA4-antiRSV3'-3'T (8281), upon twice daily administration, showed an EC₉₀ (concentration to reduce virus replication by 90%) of 0.02 μM compared with an EC₉₀ for ribavirin of 1 mM (D. Barnard, R. Sidwell, W. Xiao and P. F. Torrence, unpublished observations). When M2 RNA concentrations in RSV-infected 9HTE cells were examined using RT-PCR, approximately an 80% decrease was seen in response to a single administration of 3.3 μM spA4-antiRSV3'-3'T (8281). Under these conditions, there was no effect on concentrations of the RSV N or P mRNAs, nor was there a discernible effect on cellular glyceraldehyde 3-phosphate dehydrogenase mRNA. There was no evidence for the involvement of RNase H in the antiviral activity of spA4-antiRSV3'-3'T (8281) since spA2-antiRSV3'-3'T (8281), which cannot activate RNase L because of the dimeric 2-5A domain, showed only weak anti-RSV activity and did not cause the degradation of M2 RNA. In addition, antiRSV3'-3'T (8281), without the 2-5A domain, and an all phosphorothioate oligonucleotide to the 8281–8299 sequence, were only weakly active against RSV. In summary, these foregoing results show that 2-5A-antisense can be a potent suppressor of RSV replication through its effect on RSV M2 RNA.

The 2-5A-antisense approach to specific nucleic acid cleavage has a number of significant advantages when compared with other approaches to targeted cellular RNA degradation. First, it relies upon a nuclease activity that is endogenous and ubiquitous in mammalian cells, but is active only when bound to 2-5A. Second, the substrate specificity of RNase L appears susceptible to modulation through changes in the antisense cassette of the 2-5A-antisense chimera. Third, in contrast to a number of other strategies, the DNA:RNA hybrid formed presumably would still be susceptible to attack by RNase H also; however, DNA chain modifications, such as methylphosphonate introduction, while eliminating RNase H-catalyzed scission as a mode of degradation, would not be expected to affect 2-5A-dependent RNase activation ability. The recruitment of an entirely new and different nuclease for the targeted destruction of RNA should greatly expand the range and potential of antisense therapeutics.

6.27. Chronic Fatigue Syndrome and Chronic Myelogenous Leukemia

Chronic fatigue syndrome (CFS) is a disease characterized by persistent fatigue and cognitive dysfunction. A number of viruses have been associated with this syndrome, including human herpes virus (HHV)-6, Epstein-Barr virus, and some enteroviruses. As a previous trial with acyclovir showed no substantive clinical improvement in CFS pa-

tients, poly(I)·poly(C₁₂U) (Ampligen) was evaluated as a potential therapeutic agent (Suhadolnik *et al.*, 1994a).

A study was conducted with 15 patients with particularly debilitating CFS. Poly(I)·poly(C₁₂U) was given to all patients (Suhadolnik *et al.*, 1994b). Levels of 2-5A synthetase, 2-5A, and RNase L were measured before and after therapy, and these data were compared with healthy untreated controls. CFS patients had a significantly lower basal level of latent 2-5A synthetase ($P < 0.001$), a higher 2-5A level ($P = 0.002$), and a higher pretherapy RNase L level ($P < 0.0001$), when compared with controls. PMBC extracts also had higher 2-5A synthetase levels than controls. All PMBC extracts tested were positive for replication of HHV-6. Poly(I)·poly(C₁₂U) therapy resulted in a significant decrease in HHV-6 activity ($P < 0.01$), a decrease in 2-5A and RNase L activity, as well as neuropsychological improvements. Whether or not any of these observations are related to the finding (Chapekar and Glazer, 1988) that immune IFN and TNF can inhibit rRNA processing and 2-5A activation of RNase L remains to be determined.

Increased endoribonucleolytic activity, as well as increased RNase L activity, were observed in extracts of PMBCs isolated from patients with chronic myelogenous leukemia (CML) (Hubbell *et al.*, 1994). The rRNA cleavage assay was used to evaluate the context of both activities. Two different rRNA cleavage products could be observed. The smallest rRNA cleavage products were generated by Ficoll-purified monocytes and granulocytes from CML patients, as well as the granulocytic fraction from healthy controls. The aforementioned cleavage products were generated by endoribonucleolytic degradation of larger RNase L-derived rRNA cleavage products. Since the endoribonucleolytic activity, as measured by time course experiments, was up to 240-fold greater in CML patients with respect to controls, RNase L and the novel endoribonuclease were postulated to play a functional role in CML.

6.28. Other Biological Receptors or Roles for 2-5A?

Is the RNase L the only biochemical target of 2-5A? A number of investigations of affinity labelling with radioactive 2-5A or its derivatives and analogues have reported, in addition to the distinct labeling of the approximately 80 kDa RNase L protein, a number of other affinity-labelled bands that were present in reduced intensity. Importantly, these bands did not label if the affinity reaction was carried out in the presence of excess 2-5A, suggesting that they too were involved in specific binding to 2-5A (Krause *et al.*, 1985b; St. Laurent *et al.*, 1983; Wreschner *et al.*, 1982; Floyd-Smith *et al.*, 1982; Bisbal *et al.*, 1989). In some instances, certain such affinity-labelled products disappeared when protease inhibitors were included during cell extract preparations and manipulation (Krause *et al.*, 1985b). Some of these results presumably might be explained as our understanding of RNase L dimerization (Dong and Silverman, 1995; Cole *et al.*, 1996; Bayard *et al.*, 1994; Salehzada *et al.*,

1993) and association with other proteins (Bisbal *et al.*, 1995) continues to emerge.

The above considerations of proteolytic degradation and protein-protein associations notwithstanding, other biological and biochemical effects of 2-5A and closely related compounds have been reported. For instance, Castora *et al.* (1991) showed that calf thymus Type I DNA topoisomerase was inhibited by a number of 2',5'-oligoadenylates, with the extent of inhibition increasing with the degree of 5'-phosphorylation and the chain length. Thus, the hexamer ppp(5'A2'p)5A was the most effective, reducing relaxation by the DNA topoisomerase I at submicromolar concentration. These findings were completely corroborated in a later report (Schroder *et al.*, 1994).

An apparent anomaly of the 2-5A system is the production by the 2-5A synthetase of ppp5'A2'p5'A, which does not bind to or activate the RNase L. Nonetheless, Alarcon *et al.* (1984) reported the inhibition of VSV production by this dimeric 2',5'-oligoadenylate, possibly through an effect on early virus transcription. Subramanian *et al.* (1990) then demonstrated that ppp5'A2'p5'A was an inhibitor of the purified RNA polymerase ("nucleocapsid") complex from VSV. Under standard assay conditions, a 50% inhibition required approximately 300 μ M dinucleotide. Characteristics of this inhibition reaction were significantly different from those usually associated with the 2-5A system generally: (1) the 2',5'-linkage was not required since ppp5'A3'p5'A was equally effective; (2) only the dimer was effective with higher oligomers of greatly reduced or no inhibitory activity; (3) the dimer triphosphate was most active, the dimer 5'-monophosphate was nearly devoid of activity, and the 5'-diphosphate was of intermediate inhibitory potency.

In a possibly related study, Tominaga *et al.* (1990) found that the dimer "core" itself, A2'p5'A, added to cells without permeability treatments, could inhibit replication of both Sindbis virus and VSV through an inhibition of viral protein synthesis. Phosphorylated forms of dimer did not show such effects. It is difficult to see the connection between this report and the work of Alarcon *et al.* (1984) and Subramanian *et al.* (1990).

In a similar vein, Pivazian *et al.* (1984) reported that the dinucleotide ppp5'A2'p5'A was a potent noncompetitive inhibitor of poly(ADP-ribose) transferase activity with a $K_i = 5 \mu$ M. The activity of longer 2',5'-oligoadenylates was not reported.

Sobol *et al.* (1993b) reported on the ability of various 2',5'-oligoadenylates and their analogues to function as noncompetitive inhibitors of primer-HIV-1 reverse transcriptase complex formation. 2',5'-Phosphorothioate derivatives of the 2',5'-oligonucleotides were the most inhibitory, with K_i s approximating 7–13 μ M. 5'-Triphosphates were needed for optimum inhibition.

Rios *et al.* (1995) showed that 2',5'-oligoadenylates could inhibit rotavirus *in vitro* transcription. Inhibition increased with chain length and was potentiated for a given oligoadenylate by 5'-phosphorylation. Inhibition appeared competitive, with an apparent K_i of 156 μ M. 3',5'-Oligoad-

enylates also were inhibitory, but only hexameric or longer chain lengths were effective. Surprisingly, *in vitro* rotavirus replication could not be inhibited by 2',5'-oligoadenylates.

In view of the concentrations required for the above effects of 2-5A and its congeners on biological receptors other than the RNase L, it is unlikely that such effects would be significant under most circumstances. However, it may be conceivable that in certain situations when micromolar concentrations of 2-5A are formed, such as during replication of picornaviruses or vaccinia virus in IFN-treated cells, 2',5'-oligoadenylates may begin to affect other enzymes, such as DNA topoisomerase I. Whether or not such possible effects may have biological repercussions remains to be seen.

7. CONFORMATION OF 2',5'-OLIGONUCLEOTIDES

7.1. Base Stacking

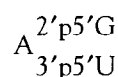
Circular dichroism (CD) studies on 2',5'-oligoadenylates showed that their hypochromicity was greater than that of 3',5'-oligoadenylates, leading to the suggestion that the degree of base stacking is greater in 2',5'-linked oligoadenylates than in 3',5'-oligoadenylates (Doombos *et al.*, 1981, 1983). On the other hand, a comparative examination of 2',5'-linked versus 3',5'-linked oligoadenylate tetramers, pentamers, and hexamers revealed that the change in extinction coefficient with growing chain length increased for the 3',5'-oligoadenylates, but remained constant for the 2',5'-linked congeners. These results implied that stacking was weaker in the 2',5'-oligoadenylates compared with 3',5'-oligoadenylates (Dhingra and Sarma, 1978). Using ^{13}C NMR, on the other hand, Schleich *et al.* (1976) noted that large chemical shift differences between the base carbons in the dimers versus the monomers indicated base stacking in both A3'p5'A and A2'p5'A. Through the tool of Raman spectroscopy, White *et al.* (1987) concluded that there was little or no base stacking in the trimeric p5'A2'p5'A2'p5'A. In contrast, Weaver and Williams (1988) found from Raman spectroscopy that p5'A2'p5'A2'5p'A was more stacked than the deoxy trimer p5'dA3'p5'dA3'p5'dA.

Studies on other 2',5'-linked oligonucleotides also differ in their conclusions regarding stacking interactions. Parthasarathy and co-workers (1982) conducted X-ray analyses of the dinucleotide A2'p5'C and concluded that while a compact right-handed helix could exist in crystals, the stacking involved intramolecular ribose O 4'-adenine stacking, rather than classical intramolecular base stacking. The dinucleotide A2'p5'U also displayed a reduced base stacking compared with A3'p5'U in another X-ray crystallographic study (Shefter *et al.*, 1969). In an X-ray study of the complex of C2'p5'A with the enzyme RNase S, no base stacking was seen in the dinucleotide since an extended structure was required to optimize contacts in the enzyme active site (Shefter *et al.*, 1969).

Kondo *et al.* (1970) investigated CD, UV, and NMR parameters associated with the isomeric pair A2'p5'A and

A3'p5'A, as well as A2'p5'C and A3'p5'C. These authors noted that a comparison of base stacking derived from a single physicochemical measurement "is not necessarily meaningful and can be quite misleading". They found that based on CD measurements, the order of base stacking was A3'p5'A > A2'p5'A and A3'p5'C > A2'p5'C. However, from UV measurements, the base stacking was in the order A2'p5'A > A3'p5'A and A2'p5'C > A3'p5'C. From NMR "dimerization shifts," the base stacking interaction was A2'p5'A > A3'p5'A and A2'p5'C > A3'p5'C, if the adenine H-8 proton of the 5'-terminal A was used to calculate "dimerization shifts". In contrast, if the adenine H-2 of the 5'-terminal A was used as a basis, then the order of stacking was A3'p5'A > A2'p5'A. Kondo and co-workers (1970) went on to develop models using NMR data to establish relative base orientations and space-filling models for backbone constraints. They concluded that base stacking interactions were stronger in A2'p5'A and A2'p5'C than in A3'p5'A and A3'p5'C, respectively. Warshaw and Cantor (1970) also concluded that 2',5'-dinucleotides possessed a significant degree of base stacking.

In a particularly compelling comparison, Sund *et al.* (1992) examined a number of branched RNA structures of the general configuration



such as that found in lariat RNA. In all cases, a clear preference was shown for 2'→5' versus 3'→5' base-stacked structures.

The consistent difference between solution studies, which mostly indicate a stronger tendency for 2',5'-oligonucleotides to base stack compared with their 3',5'-counterparts, and X-ray crystallographic studies, which indicate little or no base stacking in 2',5'-oligonucleotides, has been ascribed to the use of the free acid nucleotide form (pH ~ 4.5) in X-ray studies and the use of the salt form (pH ~ 7.5) in various solution studies (Parthasarathy *et al.*, 1982).

7.2. Association of 2',5'-Oligonucleotides with Other 2',5'-Linked Nucleic Acids and with 3',5'-RNA and DNA: Theoretical and Empirical Studies

These experimental studies have been accompanied by several relevant theoretical calculations that may provide some additional understanding of the fundamental differences between 2',5'- and 3',5'-oligonucleotides. Dhingra and Sarma (1978) employed empirical dimerization shifts collected from dinucleotides to generate a set of conformational parameters to describe 2',5'-dinucleotides in general. The most dramatic outcome of this was the finding that the glycosidic torsion angle, χ , for the first (5') residue of a 2',5'-dinucleotide was in the low *syn* domain ($\chi_1 \sim -50^\circ$), while the glycosidic torsion angle for the second residue (2') was in the *anti* domain ($\chi_2 \sim +60^\circ$). Thus, the glycosidic torsion angle, χ_1 , for the first (5') nucleotide of the

2',5'-dimer was in the *syn* range in contrast to the *anti* conformation found in the second residue of the 2',5'-dimer, and most critically, in the 3',5'-dinucleotide, which possesses an *anti* conformation ($\chi_2 = 60^\circ$). The end result of this is that when determined conformational parameters for A2'p5'A were used in a repeating mode, no helix formation could be obtained. Only when the backbone torsion angles were fixed and glycosidic torsion angles allowed to vary could helices be formed in certain domains of χ_1 and χ_2 . In short, 2',5'-dinucleotides could be incorporated into helical structures, but at such values of χ that no base stacking interactions can be formed.

Parthasarathy *et al.* (1982) found that the overall conformation of 2',5'-dinucleotides, based on their X-ray diffraction analysis of A2'p5'C, was markedly different from their 3',5'-counterparts since the 2',5'-bond oriented the backbone inwards toward the bases rather than away from the bases, as in 3',5'-linked oligos. Using conformational parameters determined for A2'p5'C, Parthasarathy and colleagues (1982) determined what possible helices might be built from 2',5'-oligonucleotides. A very compact single-stranded poly(G2'p5'C) helix could be generated readily and was characterized as a right-handed helix with four bases per helical turn with alternating purines and pyrimidines. The repeat unit was a dinucleotide, GpCp or CpGp, with the purines in the *syn* conformation and the pyrimidines in the *anti* conformation. Base stacking was minimal in the GpC moiety, but ribose-4'O stacking was important. However, in the CpG moiety, there was base stacking of C and G. In the CpG fragment, the 5'-phosphates had different conformations, as in Z DNA. Attempts to build a double helix with 2',5'-phosphodiester linkages failed because in order to keep the residues stacked, the Watson-Crick hydrogen-bonding sites had to be pointed in opposite directions.

Anukanth and Ponnuswamy (1986) performed exhaustive theoretical calculations of A2'p5'A. Analysis of over 400 probable conformations resulted in only 10 conformations within 5 kcal/mol of the global minimum energy state. As a result, Anukanth and Ponnuswamy were able to generate a double-helical structure similar in backbone torsion angles to standard A and B double helices; however, base stacking may be minimized in such a model.

Srinivasan and Olson's (1986) calculations indicated that long helices cannot form in 2',5'-duplexes, although short 2',5'-dimer miniduplexes were possible. Adjacent base stacking resulted in mixed glycosyl angles and sugar puckering in the 2',5'-ribose-phosphate backbone. Their data implied the possibility of a 2',5'-duplex with alternating A- and B-type stacking patterns.

Experimentally, the solid phase support synthesis of 2',5'-linked oligoribonucleotides has demonstrated that complementary sequences can form complexes with antiparallel Watson-Crick hydrogen bonded strands, albeit with T_m s significantly lower than their 3',5'-linked analogues (Kierzek *et al.*, 1992). Thus, the self-complementary all 2',5'-octaribonucleotide CGGCGCCG possessed a T_m of 46°C, whereas

under the same conditions, the all 3',5'-octaribonucleotide of the same sequence had a T_m of 79.3°C.

Giannaris and Damha (1993) have reported that 2',5'-linked oligoribonucleotides exhibit remarkable specificity for ssRNA; for instance, the 13-mer 2',5'-r(CCCUCUCCCUCU) formed a duplex with its 3',5'-complementary oligomer 3',5'-r(AGAAGGGAGAGGG) of T_m 40°C in 0.11 M salt (pH 7), but no detectable complex formation was observed with the corresponding DNA oligomer 3',5'-d(AGAAGGGAGAGGG). The 2',5'-decaribonucleotide 2',5'-rA₁₀ formed a complex (triplex) with poly(U) of T_m 54°C, whereas the corresponding complex with poly(dT) had a T_m of -1°C in 1 M NaCl. No complex of poly(dT) and 2',5'-rA₁₀ was observable at a concentration of 0.1 M NaCl. Giannaris and Damha also observed complex formation between 2',5'-rA₁₀ and 2',5'-rU₁₀. This displayed a T_m of 8°C in 1 M NaCl as compared with a T_m of 28°C for the corresponding all 3',5'-linked duplex 3',5'-rA₁₀: 3',5'-rU₁₀.

Recently, there has been a flurry of publications on DNA with 2',5'-phosphodiester linkages (Dougherty *et al.*, 1992; Hashimoto and Switzer, 1992; Jin *et al.*, 1993; Jung and Switzer, 1994; Alul and Hoke, 1995; Robinson *et al.*, 1995; Sheppard and Breslow, 1996), although initial reports used a confusing 2',5'-designation for the 2',5'-phosphodiester bond. The general conclusions that can be drawn from these studies are that 2',5'-DNAs can form stable double-helical structures of the A genus conformation, but of markedly depressed stability as compared with their 3',5'-linked counterparts. For instance, the self-complementary 2',5'-d(CCGGCCGCGCGC) had a T_m of 36°C in 0.1 M NaCl as compared with a T_m of 72°C for the corresponding 3',5'-d(CCGGCCGCGCGC). However, just as with the 2',5'-ribooligomers, such 2',5'-DNAs formed duplexes with RNAs of comparable stability to the 3',5'-RNA/DNA hybrids. This selectivity, together with enhanced nuclease resistance, has led to suggestions that 2',5'-DNAs may find useful antisense applications.

7.3. Additional Conformational Parameters

In a detailed comparative study using NMR and model building, van den Hoogen *et al.* (1989) explored conformational facets of the 2-5A trimer p5'A2'p5'A2'5p'A and a series of 8-brominated analogues. Substitution of br⁸A for A in the first (5') residue or second residue of p5'A2'p5'A2'p5'A caused a shift of the N/S sugar conformation equilibrium from a slight preference for N in the first and second residues to a strong N preference. Moreover, this shift to a strong preference for N was present, albeit of less magnitude, in all brominated analogues, regardless of whether the residue was brominated. For instance, the mole percent of N was 66 ($T = 25^\circ\text{C}$) in the 5'A of p5'A2'p5'A2'p5'A and was shifted to >90% N in p5'br⁸A2'p5'A2'p5'A, but was still shifted to 67–80% in p5'A2'p5'br⁸A2'p5'br⁸A and p5'A2'p5'A2'p5'br⁸A. In contrast, bromination of the third (2') residue of 2',5'-(pA)₃ led to a strong preference

for the S-type sugar conformer, whereas no such preference is seen when the third A is unmodified. Brominated A residues led to greater eclipsing of ribose H2' and H3' and flatter ribose rings. The bromination of residues A1 and A2 also led to a *syn* orientation of the bases in those residues. When the A3 residue or 2',5'-(pA)₃ was 8-brominated, the A3 base *syn* conformation was favored together with the S sugar conformation. These glycosidic conformations contrasted strongly with those in 2',5'-(pA)₃ itself, in which all sugar-base torsion angles were in the *anti* range. The variation in glycosidic torsion angle led to different stacking interactions in 2',5'-(pA)₃ as compared with the various brominated analogues.

In a related investigation of 9-(3'-fluoro-3'-deoxy-β-D-xylofuranosyl)-adenine and 3'-fluoro-3'-deoxyadenosine containing 2',5'-linked oligoadenylate trimers (van den Boogaart *et al.*, 1994), it was found that when the fluorine substituent was *xylo*, the N-type sugar conformation was favored with flattening of the ribose ring and *syn* orientations for the bases in the *xylo*-fluoro-substituted residues. When the fluorine was in the *ribo* position in 3'-fluoro-3'-deoxyadenosine-substituted analogues, the sugar conformation of the substituted residue was nearly pure S-type ribose, but fluorine had little or no effect on other conformational parameters.

8. 2-5A AND THE RNA WORLD

The discovery of ribozymes (Kruger *et al.*, 1982; Guerrier-Takada *et al.*, 1983) rekindled earlier speculations (Woese, 1967; Orgel, 1968; Crick, 1968) that RNA replication preceded protein synthesis during evolution. "The RNA world" (Gilbert, 1986; Pace and Marsh, 1985; Sharp, 1985; Lewin, 1986; Gesteland and Atkins, 1993; James and Ellington, 1995; Lazcano and Miller, 1996) encompasses the ideas that at an early epoch of evolution, RNA replication was responsible for the transfer of genes from one generation to the next, that Watson-Crick base-pairing was then, as now, the operational recognition mode between bases, and that catalytic RNAs, not proteins, were specified by the RNA genes.

From a prebiotic viewpoint, the assembly of 3',5'-phosphodiester bond-linked oligonucleotides is an implausible event unless the intervention of an appropriate catalyst is postulated, which would increase the percentage of 3',5'-bond formation (Usher, 1968). In point of fact, lead ion-catalyzed or uranyl ion-catalyzed oligomerization of activated nucleotides, such as nucleoside 5'-phosphorimidazolides, gives rise to predominantly 2',5'-linked oligonucleotides (Sleeper and Orgel, 1979; Sawai *et al.*, 1988). This sticking point in the chemical evolution of 3',5'-linked RNAs has been largely by-passed in the prebiotic field through the postulation of a yet unbound metal ion, acid-base catalyst, or specific mineral surface to which nucleotide precursors might have adsorbed, resulting in the production of the desired 3',5'-linkages (Gesteland and Atkins, 1993). Even under conditions of template-directed synthesis, 2',5'-phosphodi-

ester bond formation is favored, except under selected conditions (Joyce, 1987; Inoue and Orgel, 1981).

This knotty problem of the propensity for formation of 2',5'- versus 3',5'-phosphodiester bonds was addressed by Usher (1977), who studied the relative rates of hydrolysis of two oligoadenylates; namely, [p5'A3']₁₂ and [p5'A3']₅p5'A2'p5'A3'[p5'A3']₅. The only difference between the two oligoadenylates was the replacement of a single 3',5'-phosphodiester bond by a 2',5'-phosphodiester bond. Both of these had equivalent hydrolytic rates under alkaline conditions. Remarkably, however, the addition of 3',5'-poly(U) to the individual oligonucleotides led to a dramatic difference in alkaline hydrolysis rates: the oligonucleotide [p5'A3']₅p5'A2'p5'A3'[p5'A3']₅, with the single 2',5'-linkage, was hydrolyzed much more rapidly than was the all 3',5'-linked [p5'A3']₁₂. This led Usher to the conclusion that the 2',5'-phosphodiester linkage was 700 times less stable than the 3',5'-phosphodiester bond. Usher rationalized this difference through consideration of the mechanism of hydrolysis of nucleotide diesters (Usher and McHale, 1976). For a double-helical RNA containing 3',5'-linkages, hydrolysis through intramolecular attack of the 2'-OH on phosphorus would involve the leaving 5'-OH in an energetically less favorable "adjacent" (Westheimer, 1968) position of the trigonal by-pyramidal intermediate. On the other hand, in a double-helical RNA containing 2',5'-phosphodiester bonds, the attack of the 2'-OH would result in the more energetically favorable "in line" departure of the 5'-OH leaving group.

The foregoing experiments, however, remain incomplete since no measurement was made of the opposing situation; i.e., the inclusion of a single 3',5'-phosphodiester bond in an all 2',5'-linked oligonucleotide and its rate of hydrolysis in a complex with either a 2',5'-linked RNA or a 3',5'-linked RNA. For that matter, no studies have been carried out on the relative rates of hydrolysis of 2',5'-phosphodiester bonds in oligonucleotides complexed with complementary 2',5'-linked or 3',5'-linked oligonucleotides relative to the behavior of the 3',5'-linkages in the same situations. Until these data are available, it remains premature to draw any conclusions about the relative stability of 2',5'- versus 3',5'-phosphodiester bonds.

As described earlier in this review (Section 7), 2',5'-linked RNAs generally seem to form less stable associations with complementary 2',5'- or 3',5'-polynucleotides than do 3',5'-RNAs. Thus, it would be expected that over time and based on this duplex stability property alone, 3',5'-RNAs would be selected over 2',5'-RNAs as the basis for self-replicating RNA.

2',5'-Oligoadenylates of the 2-5A system are not the only 2',5'-phosphodiester-linked nucleic acids found in nature. Probably the best known 2',5'-phosphodiester bond is that which forms in the lariat RNA intermediate during gene splicing (Voet and Voet, 1990). A 2',5'-phosphodiester linkage also is formed in a peculiar satellite DNA called multicopy single-stranded DNA (Sun *et al.*, 1989). This multicopy single-stranded DNA was made up of a 67-

base single-stranded DNA with its 5'-terminus linked to a 58-base RNA through a 2',5'-phosphodiester bond to the 2'-hydroxyl of guanosine at the 15th nucleotide residue of the RNA.

Laurence *et al.* (1984) used a highly specific monoclonal antibody for A2'p5'A-containing oligonucleotides to demonstrate the presence of dimeric to pentameric 5'-nonphosphorylated 2',5'-oligonucleotides in a variety of mammalian tissues, but even more remarkably, in bacteria and yeast. The origin and function of the 2',5'-linked adenylylates in the latter remains unknown.

These examples notwithstanding, 2',5'-oligoadenylates remain the only example of nucleic acids that contain more than one 2',5'-linkage in tandem. This, in spite of the ready test-tube ability of 2-5A synthetase to add adenylylate in 2',5'-linkage to other substrates or to add other nucleotides in 2',5'-linkage to 2',5'-oligoadenylates themselves.

Is 2-5A a relic of a bygone RNA world? If so, why are the 2-5A synthetase and RNase L not found in simpler life forms, such as bacteria, fungi, molds, yeasts, amphibians, and insects (Cayley *et al.*, 1982b)? The report (Kuuskalu *et al.*, 1995) of a functionally active 2-5A synthetase in marine sponge suggests that the entire question of the presence of the 2-5A system in such more "primitive" animals should be revisited. Or might 2-5A be simply a later evolutionary adaptation of 3',5'-RNA? It may be possible that the eventual determination of the role the 2-5A molecule plays in the enzymic action of RNase L may provide an answer. Specifically, does the 2-5A molecule itself actually participate in the catalytic reaction? Or is the cleavage preference of RNase L for UpNp sequences related to hydrogen bonding interactions between substrate and 2-5A in the recognition process (Floyd-Smith *et al.*, 1981)?

9. CONCLUSION

The 2-5A system is a unique and powerful regulatory system. Thus, dsRNA stimulates a unique enzyme to synthesize small and novel 2',5'-phosphodiester-linked oligonucleotides that, in turn, activate a unique nuclease, RNase L. Insofar that the decay of mRNA is of cardinal importance in the post-transcriptional regulation of gene expression and can be influenced by a variety of environmental and developmental factors (Belasco and Brawerman, 1993), it seems clear that the potential roles of the 2-5A system could be widespread. Indeed, past research in this field has broached a large number of arenas in which a role for the 2-5A system has been posited; nonetheless, the only generally accepted role for the 2-5A system to date is as one mediator of the anti-picornavirus effects of IFN. Research in the next decade likely will reveal the answers to many questions posed in the 20 years since 2-5A was discovered. For instance, major advances may be expected in our understanding of the key enzyme RNase L. A complete picture of how 2-5A acts to turn on this nuclease certainly is obtainable now that pure enzyme is accessible. An intriguing question will be whether RNase L and/or the lessons it will

teach us can eventually be adapted to be of benefit in human medicine. This would bring the story full circle from IFN itself to a therapeutic based on one aspect of IFN action. The question remains whether a two-edged sword can be shaped to a delicate scalpel.

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