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INTERFERON ACTION: A. STUDIES OF THE EFFECT OF INTERFERONS ON SV40 REPLICATION. B. STUDIES OF THE 2'-5' A SYSTEM.

A Dissertation
Presented to the Faculty of the Graduate School
of
Yale University
in Candidacy for the Degree of
Doctor of Philosophy

by
Mariano Agustín García Blanco
May 1988

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ABSTRACT

INTERFERON ACTION: A. STUDIES OF THE EFFECT OF INTERFERONS ON SV40 REPLICATION. B. STUDIES OF THE 2'-5' A SYSTEM.

Mariano Agustín García Blanco

Yale University
1988

This thesis is divided into two parts: the first deals with studies that focus on the effect of interferons on the expression of foreign genetic material. The second part deals with the interferon-regulated (2'-5')oligoadenylate system. This system is most probably an effector arm of the anti-viral anti-mitogenic actions of interferons.

I show here that interferons inhibit the expression of both early SV40 RNA and SV40 Tag, and SV40 DNA replication in SV40 infected cells. Interferons selectively inhibit the expression of the Tag encoded by superinfecting SV40 virions in SV40-transformed cells superinfected with SV40. The effect of interferon was seen when cells were transfected with SV40 DNA or infected with SV40 virions.

Data shown indicates that interferons inhibit the expression of bacterial genes driven by the SV40 early promoter-enhancer elements. Moreover, interferon treatment inhibits the expression of

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a bacterial gene driven by cellular promoter-enhancer elements, when newly transfected into cells. Thus, it seems that interferons can distinguish between stably integrated genes and newly arriving genes.

The (2'-5') oligoadenylate system is composed of the (2'-5') oligoadenylate synthetases, the RNase L, a (2'-5')phosphodiesterase, and the (2'-5')oligoadenylates. In the presence of double stranded RNA the (2'-5')oligoadenylate synthetases convert ATP into (2'-5')oligoadenylates. The RNase L is a latent endonuclease that when activated by binding a (2'-5')oligoadenylate cleaves on single stranded regions of RNA. A scheme for the partial purification of the RNase L from calf spleens is presented. Furthermore, the discovery of a (2'-5')oligoadenylate binding protein in wheat is described.

Growth conditions profoundly affect the (2'-5')oligoadenylate system. I show here that Platelet-derived growth factor increases the levels of (2'-5')oligoadenylate synthetase transcripts and of (2'-5')-oligoadenylate synthetases in Balb/c-3T3 cells. Moreover, Platelet-derived growth factor decreases the levels of RNase L in these cells. Interferon treatment inhibits the mitogenic action of Platelet-derived growth factor without inhibiting the expression of c-myc RNA.

Finally I present a hypothesis implicating endogenous double stranded RNAs in the control of cell growth.

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To my parents, María Teresa Blanco Cestero and Jose María García Madrid, for their total support.

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To Olivia and Rebecca García Putnam.

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ABBREVIATIONS

2'-5'A, (2'-5')oligoadenylates; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; IFN(s), interferon(s); MOI. multiplicity of infection; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; pCp, (3',5') cytidine bisphosphate; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonylfluoride; ppp(A2'p)$_n$A, where n = 1 to 15, represents oligonucleotides of the general structure given by 5'triphospho-oligo[(2'-5')adenylyl]adenosine; PPP, platelet poor plasma; polyI:polyC, polyinosinic acid: polycytidylic acid.SV40 simian virus 40; Tag, T-antigen; TK, thymidine kinase; XGPRT, xanthine-guanine phosphoribosyltransferase.

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CHAPTER ONE

INTRODUCTION: A SELENITE'S* VIEW OF THE INTERFERON SYSTEM.

Interferons. Interferons (IFNs) are proteins that were first recognized for their ability to convert cells to the anti-viral state: a reversible state in which cells are poor hosts for viral multiplication (1). Among other activities IFNs inhibit the growth of cells (1).

Originally discovered in 1957 by Isaacs and Lindenmann (2,3), IFNs are still the subject of intense study. Human IFNs are encoded by three distinct gene families: at least 15 genes are members of the human alpha IFN gene family, the human ß1-IFN gene, the ß2-IFN gene and possibly others are included in the beta IFN gene family. One gene encodes a human gamma IFN(4). ß2-IFN has low activity in anti-viral assays, and its sequence is homologous to a B-cell growth factor (5-7); it is debatable whether or not this compound is an interferon.

Many other IFNs have not been well characterized, a brief

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* Selenites are keen observers of earthly systems, but being so distant, however, their observations are sometimes sketchy.

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and probably incomplete list follows: 1) The so called endogenous IFNs found in cell cultures not exposed to known inducers of IFNs (8-10); 2) IFNs induced by growth factors (11-16); 3) placental IFNs (17), 4) acid labile alpha IFNs (18), and IFNs induced by lentiviruses (18b). Given the many different IFNs, their differential expression, and their slightly different activities, It is reasonable to assume that IFNs will play many and varied physiological roles (1,19-21).

Upon viral infection, most cells produce IFNs. This requires the synthesis of IFN mRNAs and the synthesis and secretion of IFNs (1,22). Secreted IFNs bind to plasma membrane receptors found in most cells (alpha competes with beta for binding and vice versa, gamma does not compete with either) (23). The receptor-IFN complex is internalized and after a lag period, in which RNA and protein have to be newly synthesized, one detects the formation of the anti-viral state (1,22,24,25).

IFNs can also inhibit cell growth (1,26-27), activate immune mechanisms (28), inhibit parasite growth (29), mediate specific antitumor effects (30), and induce changes in the cytoskeleton (1).

Regulation of interferon expression. Many diverse agents induce IFN expression (1,22). I will classify these into two general categories: 1) IFN inducers that are, contain or induce the expression of a dsRNA ; and 2) inducers that do not contain a dsRNA component and have not been shown to induce expression of a dsRNA.

The first category is the best studied, it includes natural (reovirus RNA) and synthetic (polyinosinic:polycytidylic acid) dsRNA , which are among the most potent inducers of IFN (31), dsRNA

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viruses, ssRNA viruses with dsRNA intermediates in the viral life cycle (1,32), and finally some DNA viruses such as vaccinia virus that induce the formation of dsRNA (33).

The second category is heterogenous; it includes viruses that have not been shown to have or induce dsRNA (1), other micro-organisms (1), growth factors and mitogens(11-16,34,35). The latter was first observed when lymphocytes were stimulated with mitogens or antigens (35,36). Several viral proteins have been implicated as IFN inducers: The C-protein of Sendai virus induces IFN (37) and trypsin digested adenovirus is infective but does not induce IFN (reviewed in ref. 1).

The mechanisms of IFN induction by dsRNA have been widely studied (38-44). The induction of the human ß1-IFN gene by polyI:polyC has been studied by Maniatis and his colleagues (40-42); their analysis has identified cis-acting elements 5' to the ß1 gene involved in its transcriptional activation by polyI:polyC (40,41). Transcription of this gene seems to be under negative and positive control , and activation requires one or more IFN-induced factors (42). Other groups studying the human ß1 gene have concluded that induction by dsRNA may also involve post-transcriptional mechanisms (43,44).

Interferon induced genes, proteins and activities. i) IFN inducible genes. The anti-viral action of IFN requires new RNA and protein synthesis; furthermore exposure to IFNs increases the levels of a large number of gene transcripts, proteins and enzymatic activities (45). Of the many genes activated by IFN (45-60); some encode characterized products (eg; the class I and II

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histocompatibility antigens (56), the (2'-5') oligoadenylate synthetases (55) and metallothionein II (48)), and others encode products of unknown activity and function (eg; the murine 202 gene (47,52,59)). The murine Mx gene encodes a protein of unknown activity but known function : it is a required factor in the IFN mediated resistance to influenza virus(53).

The induction of these genes is complex, most likely involving transcriptional and post-transcriptional mechanisms (46,48,50,52,54,56,58,59,59b). A DNA segment, spanning 5' flanking sequences of the murine 202 gene and including a sequence common to many IFN inducible genes (48), confers IFN inducibility to a heterologous gene, moreover this activity resides in an enhancer element (52, Gribaudo and Lengyel, unpublished data).

ii) IFN induced proteins and enzymatic activities. IFNs induce the expression of many proteins (61,28): membrane proteins such as the histocompatibility antigens (62) and the Fc receptor (62), dsRNA binding proteins (61,63) and secreted proteins (63). As with gene activation, alpha, beta and gamma IFNs induce the expression of overlapping but not identical sets of proteins (61).

Many enzymatic activities are induced by exposure of cells to IFN: indoleamine 2,3-dioxygenase (64), nucleoside diphosphate kinase (64b), the (2'-5') oligoadenylate synthetases (65,66), the 2'-5'A dependent endonuclease (RNase L) (22,67), a 2'-5'A phosphodiesterase (68), and the dsRNA dependent protein kinase (69-71).

dsRNA dependent activities regulated by IFNs. i) The dsRNA dependent protein kinase. An IFN inducible dsRNA dependent

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protein kinase was discovered by Lebleu et al (69), Zilberstein et al (70) and Roberts et al (71). The activated kinase phosphorylates the alpha subunit of the eukaryotic translation initiation factor 2, eIF-2, and a 68 kD protein (72). Phosphorylation of eIF-2 blocks the initiation of protein synthesis (72,73) by sequestering the reversing factor (RF, also designated GEF) that normally catalyzes the dissociation of eIF-2•GDP (74). The phosphorylated 68kD protein is thought to be the kinase (75).

The level of the kinase is induced as much as ten fold by IFN, and its activation is exquisitely sensitive to dsRNA; low doses of dsRNA activate it, high doses inhibit the enzyme (72). This activity has been detected in IFN treated virus infected cells and it is considered one of the mediators of the anti-viral state (76). When mouse 3T3 fibroblasts were grown in culture the level of the kinase was seen to rise as the cells approached confluency, furthermore this was shown to be due to secretion of IFNs into the media (77). The role of the kinase in the IFN mediated control of cell growth is unknown.

ii) The 2'-5'A system: the (2'-5')oligoadenylates, the enzymes that synthesize them and the enzyme(s) that degrade(s) them. Kerr and colleagues discovered an IFN inducible, dsRNA dependent, activity that converts ATP into oligoadenylates of the general formula (2'-5')$p_xA(pA)_n$ (65,66). This activity is now referred to as (2'-5')oligoadenylate synthetase. (2'-5')(p)ppA$(pA)_n$, with $n > 1$ will be designated 2'-5'A. 2'-5'A bind and activate a latent endonuclease, RNase L (22,78). However, 5'-monophosphate or 5'-OH (2'-5') oligoadenylates bind but do not activate the

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endonuclease (79). These competitive inhibitors may play a physiological role in vivo by regulating the activation of the RNase L (80).

Cytoplasmic (2'-5')oligoadenylate synthetases have been purified to homogeneity from mouse EAT cells and human HeLa cells and extensively charaterized (81,82). The induction of the (2'-5')oligoadenylate synthetases (synthetases) involves induction of the mRNAs coding for these proteins (83,84). Several different synthetase activities encoded by different mRNAs have been identified (55,84,84b) including a synthetase activity in the cell nucleus, which in EAT cells is different from the cytoplasmic synthetase (84). A gene encoding human (2'-5')oligoadenylate synthetases has been isolated revealing the occurrence of differential splicing as a mechanism to generate diverse synthetases(55).

The synthetases can be induced more than 200 fold by IFNs (22,78). Other agents and conditions can also induce this activity: steroids (85), steroid withdrawal (86), cell growth arrest (87,88) and paradoxically growth factors, PDGF and EGF (13,89). Furthermore a 2'-5'A synthetase mRNA and a 2'-5'A synthetase activity were shown to fluctuate in level with the cell cycle (10).

A phosphodiesterase that preferentially cleaves 2'-5' bonds was detected by several groups, Revel and coworkers showed that this enzyme was modestly induced by IFN treatment of L cells (68) and generously during ConA stimulation of lymphocytes (90,91).

<u>iii)The 2'-5' A system: the 2'-5'A dependent endonuclease (RNase L) and other 2'-5'A binding proteins. a) Discovery of the</u>

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endonuclease. The first report of an IFN induced nuclease activity was published by Marcus et al in 1975 (92), however, this activity was not characterized. The discovery of the RNase L was made by Lengyel and colleagues in 1976 (93). These authors showed that cytoplasmic extracts from IFN treated EAT cells degraded reovirus mRNA faster than extracts from control cells, but only if contaminating reovirus dsRNA was present in the incubations (93,94). This group also demonstrated that reovirions isolated from IFN treated cells contain an associated endonuclease activity (95). The activation of the EAT endonuclease, RNase L, requires incubation of the extracts with ATP and dsRNA; however after a short incubation, ATP and dsRNA can be removed without impairing the activity (94,96). The RNase L was shown to be present in many cells and to cleave different RNAs with different efficiencies, dsRNA was not digested by this activity (97,98).

Ratner et al showed that dsRNA and ATP are required to synthesize a low molecular weight substance that activated the endonuclease; furthermore they showed that this substance behaved like 2'-5'A (99). Baglioni et al (100) and subsequently others (101-105) showed that authentic 2'-5'A activates this endonuclease.

No selectivity of this endonuclease for viral over cellular RNAs could be demonstrated in vitro, whereas in IFN treated virus infected cells a selective degradation of viral RNAs was shown in the case of reovirus (106). In order to explain this, Baglioni and colleagues proposed the following: In vivo the 2'-5'A synthetase would bind to dsRNA in viral replicative complexes and hence becomes activated, synthesizing 2'-5'A that would activate the

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RNase L locally. This would, in turn, preferentially degrade neighboring RNA, that is viral RNA (100).

Several data support this hypothesis. Both reovirions made in IFN treated cells (95), and mengo virus replication complexes isolated from IFN treated cells (107) have been shown to contain associated ribonucleases . Furthermore Nilsen and Baglioni showed that in extracts of IFN treated HeLa cells, mRNA covalently linked to dsRNA was preferentially degraded over mRNA not covalently linked to dsRNA and this effect was inhibited by agents known to inhibit the synthetase (108,109).

The inhibition of in vitro protein synthesis by dsRNA is attributed to the activation of the dsRNA dependent protein kinase and of the 2'-5'oligoadenylate synthetase-RNase L pathway (104,109,110). Several groups attempted to proved that these mechanisms were operative in vivo. When 2'-5'A or dsRNA is introduced into cells, protein synthesis is inhibited and a ribonuclease activity is detected suggesting that the above pathways, elucidated in cell extracts, are active in vivo (22,76,111-113). Other studies linking these pathways to the anti-viral effects of IFNs will be reviewed below.

b) Biochemical characterization of the endonuclease.

Slattery et al demonstrated a tight binding between 2'-5'A and RNase L by the selective retention of enzyme bound labeled 2'-5'A, but not free labeled 2'-5'A, on nitrocellulose filters (114). Knight et al modified this binding assay by using (2'-5')pppApApApA[32P]pCp, a labeled analogue synthesized by ligating 2'-5'pppApApApA and pCp with T4 RNA ligase (115). This binding assay can be used to

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measure levels of RNase L and of 2'-5'A (115). Antibodies against many epitopes in the 2'-5'A molecule have been obtained and can complement the 2'-5'A binding assay in the detection of 2'-5'A and 2'-5'A-like products (115,116).

Kerr and coworkers developed a method to selectively crosslink a periodate oxidized analogue of (2'-5')pppApApApA[32P]pCp to RNAse L. The crosslinked product from cytoplasmic extracts of HeLa cells migrated on SDS-PAGE gels as a protein of 80 kD (117-118). Floyd-Smith et al used UV light to crosslink (2'-5') pppApApApA[32P]pCp to cytoplasmic extracts of EAT cells and identified a 77 kD protein (119). The crosslinked protein, the 2'-5'A binding activity and the 2'-5'A dependent endonuclease activity all co-purified through several steps (118,119) and therefore will all be referred to as RNase L.

RNase L was shown to cleave poly U but not the other common ribohomopolymers (120), the preferred cleavage sites of the RNase L are 3' to UAp, UGp and UUp on LaCrosse encephalitis virus and influenza virus RNAs (121) and also on the bacteriophage R17 RNA (120).

2'-5'A binding activity was detected in all mammalian tissues and cells studied (118,122,123,124), in reptilian tissues and at low levels in amphibian tissues, but not in fish,insect,plant, slime mold or bacteria (124). The 2'-5'A binding activity, a 77-80 kD crosslinked protein and RNase L activity were found in the cytoplasm and nuclei of several mammalian cell lines (119,136). In addition an EAT cell nuclear salt wash was shown to contain several crosslinked proteins, many of which bound 2'-5'A with very high

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affinity and some of which were IFN inducible (84).

Several schemes for the partial purification of the RNase L have been published (102,118,119,125). A low titer rabbit antiserum to the endonuclease was obtained and used to identify the protein on Western blots (126). Unfortunately the antibody is in short supply given the untimely death of the animal when the titers began to rise (C. Dieffenbach, personal conmmunication). The implications of this have not escaped us (127).

c)Evidence for the involvement of the RNase L in the anti-viral actions of IFNs. The evidence is the following: 1) The inhibition of EMCV RNA expression in IFN treated cells correlates well with the level of synthetase activity detectable in cell extracts (128); moreover 2'-5'A and RNase L activity are detected in IFN treated, EMCV infected cells (129-132). Furthermore in IFN treated reovirus infected cells, an inhibition of the expression of viral mRNAs (106) is accompanied by the synthesis of 2'-5'A and the activation of the RNase L (133).

2) A second line of evidence comes from the study of the NIH-3T3 clone 1 cells, which have normal levels of functional synthetase and dsRNA dependent kinase, but low levels of the RNase L (134-136). This cell line is inefficiently protected from many viruses by IFN treatment, and does not respond to introduced 2'-5'A (134,135). 3T6-VR cells, however,which constitutively secrete IFN, and have undetectable levels of RNase L were reported to be resistant to virus infection (137). This contradiction has not been resolved.

3) A third line of evidence is based on studies using

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inhibitors of the enzymes of the 2'-5'A system (138-140). In vitro cleavage of nascent reovirus mRNAs in extracts from IFN treated cells is inhibited by incubating these reactions with either 2'dATP, an inhibitor of the synthetases, or (2'-5')pApApA(3')OCH_3, a competitive inhibitor of the RNase L (139). Furthermore another very stable competitive inhibitor, (2'-5')CH_3S(5')ApApA(3')OCH_3, inhibits the activation of RNase L in cell free systems and in intact L_{929} cells (140). In IFN treated EMCV infected L_{929} cells it inhibits rRNA cleavage and diminishes, albeit modestly, the anti-viral effect of IFN (140).

d) The evidence linking the RNase L to the control of cell proliferation. I will briefly summarize the evidence, mostly circumstancial, that the RNase L is linked to the control of cellular proliferation:

1) The level of detectable RNase L is low in actively growing JLS-V9R or NIH-3T3 cells, but can be induced by the growth arrest mediated by confluence (141-143). IFN can also induce the levels of RNase L in these cell lines; however, IFNs are apparently not involved in the growth arrest mediated effect (142,143).

2) Differentiated embryonal carcinoma cell lines have detectable levels of RNase L whereas undifferentiated embryonal carcinoma cell lines do not (144). Furthermore, when an undifferentiated cell line (PC13) differentiates in culture the expression of RNase L is induced (144).

3) During embryogenesis in the mouse, brain tissue shows a dramatic induction of RNase L levels from undetectable early in the process to high in the full term animals (145).

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Anti-viral effects of IFN. i) General considerations. The anti-viral actions of IFN have been intensely studied, yet there is no single case in which a definite mechanism has been proven to be the(a) mediator of the anti-viral effect. Three basic questions need to be answered for each virus-cell-IFN system: 1) What is(are) the steps, in the viral life cycle, which is(are) primarily inhibited by IFNs?, 2) what are the cellular factors involved in the inhibition?, and 3) what are the mechanisms of the inhibition? Rather than review every paper on every virus ever inhibited by IFN, a gargantuan task, or superficially touch on some examples, I will summarize below the known facts and the speculative ideas on the effect of IFN on one virus: Simian virus 40. Not only is this relevant to the work presented in chapters 2 and 3, but it also provides an idea of the complexity of the anti-viral effect of IFN.

ii) IFNs and Simian virus 40. a) The effects of IFNs added prior to SV40 infection. The first published studies on the effects of interferons on SV40 were by Todaro and coworkers (146,148). Preparations of human alpha IFNs were shown to inhibit SV40 transformation of mouse 3T3 fibroblasts in culture with the same potency with which the IFNs inhibited VSV plaque formation (147,148). These authors showed that: 1) in order to obtain maximal inhibition of transformation, IFNs have to be added before infection , 2) IFN treatment does not inhibit virally stimulated cellular DNA synthesis, and 3) if the 3T3 cells are growth arrested in low serum IFN inhibits transformation when added after infection (147-148).

Oxman and several collaborators published a series of papers between 1966 and 1977 that were germinal to this field.

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Their findings were as follows: 1) Alpha IFNs added only before SV40 infection delay the appearance of Tag and reduce the number of cell staining with an immunofluorescent anti- Tag antibody. This was shown in mouse and monkey cells with mouse and human alpha IFNs respectively (149,150). 2) The inhibition of transformation and of Tag staining follows the same IFN dose response curve, with 1 unit of mouse alpha IFN giving 50% inhibition (149). 3) IFNs added 4 hours post infection have no effect on either Tag staining or transformation (149). 4) IFNs added before infection inhibit the accumulation of early SV40 RNA but not cellular RNA. The latter was done in Vero (African green monkey kidney) cells which were treated with cytosine arabinoside (Ara-C) to prevent SV40 DNA synthesis and therefore insure that this inhibition was not secondary to inhibition of viral DNA synthesis (151). 5) Furthermore, the nuclear and cytoplasmic pools of SV40 RNA are both decreased (152), and isolated nuclei from IFN treated, SV40 infected Vero cells transcribe several fold less SV40 RNA than do nuclei isolated from SV40 infected cells (153). Interestingly this effect was abrogated by incubation of the nuclei in high salt, evidence that the SV40 DNA was present in the isolated nuclei preparation but transcripts could not accumulate (153). 6) Cycloheximide treatment did not mimick the effect of IFN, suggesting that early transcription does not require protein synthesis, further suggesting that IFN treatment affects the expression of viral RNA directly (148).

The inhibition of SV40 RNA expression in IFN treated SV40 infected cells was also reported by other laboratories (154-157).

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Graessman et al showed that when IFN treated cells are microinjected with SV40 RNA, the level of Tag is lower than in microinjected cells not exposed to IFN (158).

The above data can be explained by invoking two distinct actions of IFN, one inhibiting expression of SV40 RNA and a second inhibiting translation of SV40 RNA. On the other hand a fast degradation of SV40 RNA could explain all the data.

Revel and coworkers added three interesting observations to the work mentioned above. These are: 1) when CV-1 (African green monkey kidney) cells are treated with monkey IFN there is a selective inhibition of the expression of Tag, SV40 early RNA, host histone, and histone mRNA (159,160). This observation is particulatly interesting in light of the fact that SV40 induces histone synthesis and that newly synthesized histones may bind to the newly uncoated virions (162-166). The IFN effect on histones is also seen in uninfected cells and may reflect a lower number of cells in the S phase of the cell cycle (167). 2) In IFN treated SV40 infected cells the SV40 19S RNA, present in low levels, is undegraded (160). 3) In these cells the level of transcriptional complexes isolated is reduced (160).

Mozes and Defendi determined the time course for the formation of the "antiviral state" measured by the inhibition of Tag accumulation. They showed that there is a complete block of Tag accumulation when IFNs are added to the medium 24 h prior to infection. Addition at later times diminishes this effect until it is undetectable when IFNs are added 16 h post infection (168).

These studies limited the possible mechanisms that could

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mediate the effect on early RNA accumulation to inhibition of one of the following:1) adsorption and/or entry of SV40 into cells and/or nuclei; 2) viral uncoating; 3) transcription of the uncoated viral chromatin; or to activation of an activity that would labilize SV40 nuclear RNA.

Oxman et al and others showed that IFN treatment has no effect on viral adsorption or on viral entry into cells and nuclei (152,156). Furthermore they concluded that in IFN treated cells, SV40 undergoes at least partial uncoating, by following the DNase sensitivity of SV40 DNA after infection (152).

Yamamoto et al showed that pretreatment with IFNs only weakly inhibited SV40 DNA synthesis and expression of Tag when SV40 DNA was transfected into cells by the DEAE dextran method (156), and later Brennan and Stark showed no effect on SV40 RNA accumulation after transfections (169). On the other hand, Mozes and Defendi demonstrated that IFN treatment inhibited in a dose dependent fashion, the accumulation of SV40 RNA and of Tag when SV40 DNA was transfected into cells (168). These authors used both the calcium phosphate (170) and the DEAE dextran (171) methods with similar results. These last data are consistent with a block to the viral life cycle at a step shared by both transfected SV40 DNA and infected SV40 virions; that is a step after uncoating.

The only attempts to measure the degradation rates of SV40 RNA were published by Samuel and coworkers in one of a series of papers on the effect of IFN on SV40 infection (172-174). The authors found no difference in the decay rates of SV40 RNA in IFN treated vs control cells. However, because of the very long pulses

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used to label the RNA it was impossible to rule out a very fast degradation of RNA in IFN treated cells (173).

b) The effect of IFN added during the late phase of SV40 infection. The effects of IFNs on the late phase of SV40 infection has been studied by Revel and coworkers. Their conclusions are as follows: 1) IFNs added 24 h post infection are as inhibitory as IFN pretreatment in terms of viral yield (159), however, others have suggested this is an effect on progeny virus (168); 2) IFNs added 24 h post infection inhibit ongoing SV40 DNA synthesis (159), 3) but no reduction of SV40 RNA and no active degradation of SV40 RNA is seen (157,160). 4) In IFN treated cells SV40 RNAs are internally overmethylated (160,176). 5) Under these conditions SV40 RNA, but not cellular RNA, is associated mainly with light polyribosomes and 80S ribosomes; and in vitro translation of these RNAs reveals a 60-70% effficiency compared with SV40 RNA from infected cells not exposed to IFN (157). 6) In SV40 infected IFN treated cells VP-1 accumulates to levels that are only 15-20% of control levels (160). 7) Degradation of 28S rRNA, preferrentially in 80S ribosomes, is detected (177,178). 8) Cytoplasmic extracts of cells infected with SV40 and treated with IFN have a dominant translational inhibitor which shuts down translation when added to in vitro translation systems (160).

It was later shown by others that CV-1 cells infected with SV40 and treated with IFNs contain high levels of 2'-5'A-like compounds, whereas control cells, SV40 infected cells or uninfected, IFN treated cells have only low levels of 2'-5' A -like compounds (179-181). Interestingly these 2'-5'A-like compounds

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detected in SV40 infected cells, bind but do not activate the RNase L (181).

c) Effect of IFNs on SV40 transformed cells. Oxman and coworkers showed that in SV40 transformed mouse 3T3 fibroblasts the expression of Tag was not inhibited by IFN even if continuosly added for 100 cell doubling times (149,150). These cells were shown to respond to IFN as measured by protection against the cytopathic effect of VSV (149,182). This was later repeated in SV40 transformed monkey cells where further infection by SV40 was inhibited by IFN (151,156). Furthermore in a SV40 transformed mouse cell line (H6-15) IFN treatment did not inhibit the expression of the endogenous Tag but inhibited expression of a superinfecting polyoma Tag (175).

iii) Anti-IFN systems of viruses. Viruses encode functions capable of inactivating some of the mediators of the anti-viral state.

Several viruses inhibit the dsRNA dependent kinase. Vaccinia virus infected cells contain an activity know as 'specific kinase inhibitory factor' (SKIF) (183) In addition it has been shown that vaccinia virus can rescue several viruses from the anti-viral effects of IFN (184). The adenovirus VA I RNA blocks the activation of the dsRNA dependent protein kinase. Studies with adenovirus mutants have demonstrated the importance of this short RNA in the translation of adenovirus late proteins (185,186). Finally influenza virus infected cells contain an inhibitor of dsRNA dependent kinase activation (187).

The 2'-5'A system is also inhibited by several viruses. The

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level of detectable RNase L, measured by the binding and nuclease assays, diminishes promptly after EMCV infection unless the cells have pretreated with IFN (117,118). Infection of cells with Herpes simplex I virus (188), SV40 (181) or vaccinia virus (189), results in the synthesis of 2'-5'A-like compounds that bind but do not activate the RNase L. A vaccinia virus mutant (ts22) has an abortive infection cycle at the non-permisive temperature (189b). There is data implicating activation of the RNase L in this viral growth arrest (189b). Furthermore, recent data suggest that levels of 2'-5'A are higher in cells infected with ts22 at the non-permissive temperature (R. Condit, personal communication).

T cell mediated cytotoxicity of virally infected cells is restricted by histocompatibility antigen recognition (190). IFNs are well known inducers of the expression of these antigens (62) and this induction has been shown to correlate with more efficient cell killing (191). Transformation of mouse cells with adenovirus 12 diminishes the expression of class I histocompatibility antigens by over 95% (192) .

Finally recent data show that expression of the adenovirus early region 1A and1B products inhibits the antiviral effect of human alpha-2 IFN on VSV in A549 cells (193).

The anti-proliferative action of IFNs. i) general comments. An inhibition of cell growth by IFNs was first demonstrated by Paucker et al (26). The literature on this topic is extensive, confused and inconclusive; a stimulating review, old but not outdated, was written by Ion Gresser in 1977 (34). More

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recently the field was reviewed by Clemens and McNurlan (266).

ii) The PDGF Balb/c-3T3 system. We will briefly focus our attention on the effect of IFN on the mitogenic stimulation of quiescent mouse fibroblasts by Platelet-derived growth factor. Tominaga and Lengyel (63) and later others (13) showed that exposure to pure mouse ß IFN inhibits the mitogenic effect of PDGF on quiescent Balb/c-3T3 cells . The inhibition requires long exposures to IFN before addition of the mitogen, and interestingly does not affect the level of c-myc mRNA (63). Kimchi et al have seen inhibition of entry into S phase when quiescent Balb/c-3T3 cells are treated with an alpha/beta IFN preparation even when this IFN is added for very short times (194). This effect was accompanied by marked inhibition of the expression of several PDGF-induced genes (c-myc,c-fos, ODC) (195).

In a related body of work Jonak et al and Lebleu et al have shown that IFN treatment decreases the level of c-myc mRNA in Daudi cells, and furthermore, that this action involves destabilization of this transcript (197,198). Kimchi et al have also seen the decrease in c-myc mRNA; however, this was not accompanied by a destabilization of the c-myc transcript, but rather, by a transcriptional shutdown (196).

The controversy in the field may be due in part to different cell growth methods and/or to the use of different and in some cases impure IFN preparations.

This thesis. I present data on the inhibitory effect of IFN treatment on SV40 viral infection, showing that this is selective for infecting SV40 virions but not for integrated SV40 genomes

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(Chapter 2). I further show that this inhibition is also seen when SV40 genes are introduced in the form of naked DNA by transfection. Moreover, I demonstrate that IFN treatment inhibits the expression of newly introduced genes viral and non-viral (Chapter 3).

In the second part of this thesis I present work on the 2'-5'A system. I present data on 2'-5'A binding proteins found in mammalian extracts (Chapter 4). I also show a scheme to partially purify RNase L from calf spleens (Chapter 4). I show the surprising discovery of a 2'-5'A binding protein in wheat (Chapter 5). I present data on the effects of mitogenic stimulation by Platelet-derived growth factor (PDGF) on the 2'-5'A system (Chapter 6). I present a hypothesis: PDGF induces the expression of endogenous dsRNAs, possibly encoded by repeated elements in the genome, that 1) trans-activate dsRNA inducible genes (eg. c-myc and ß1-IFN), and 2) activate the (2'-5')oligoadenylate-RNase L pathway. I mention preliminary data in favor of this hypothesis (Chapter 7). Finally I will summarize the basic findings in this thesis and will take a look at the future of the IFN field (Chapter 8).

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CHAPTER TWO

SELECTIVITY OF INTERFERON ACTION IN SIMIAN VIRUS 40 TRANSFORMED CELLS SUPERINFECTED WITH SIMIAN VIRUS 40.

INTRODUCTION.

Simian Virus 40 (SV40), a double-stranded DNA virus belonging to the papovavirus family, can replicate in, and lyse monkey cells (161). SV40 cannot replicate in mouse cells and can replicate only in some human cells (161). In the majority of the cases studied , SV40 infection of mouse and human cells results in the accumulation of SV40 specific early RNAs and proteins, but SV40 DNA is not efficiently replicated and SV40 specific late RNAs and proteins do not accumulate in detectable amounts(161). SV40 can transform human and mouse cells. The viral genome (or an appropriate part of it) is integrated into the cellular DNA of the transformed cells, and SV40-specific early RNAs and proteins accumulate (161).

In monkey cells exposed to IFNs before infection with SV40 , there is a strong inhibition of SV40 replication and cell lysis (151,155,160,168,169,173). When mouse or human cells are exposed to IFNs before infection with SV40, the accumulation of SV40-

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specific early RNAs and proteins is inhibited (146,151,156,168,182). However exposure of SV40-transformed mouse or human cells to IFNs does not result in a decrease in the accumulation of the early products (146,156,175,182). This is not a consequence of a general unresponsiveness of these cells to IFNs; if the SV40-transformed mouse cells are treated with IFNs before infection with vesicular stomatitis virus (VSV), the cells are protected from the cytopathic effect of this virus (182). Moreover, if the cells are treated with IFNs before infection with polyoma virus, the accumulation of polyoma Tag is inhibited (175).

Here we show that pretreatment with IFNs 1) inhibits the accumulation of Tag and SV40 RNA in monkey cells, and 2) selectively inhibits the expression of the viral Tag, but not the endogenous Tag, in SV40 transformed cells superinfected with SV40.

MATERIALS AND METHODS

IFNs. A human alpha IFN preparation from buffy coats (specific activity, 106 NIH mouse IFN reference units per mg of protein) was obtained from Biotechnologies, Inc. Hartford, Ct. and pure recombinant human alpha-2 IFN (specific activity, 1.5 x 108 NIH mouse IFN reference units per mg of protein) was obtained form Schering Corp., Bloomfield, N.J. Electrophoretically pure mouse mouse ß IFN (specific activity , 109 NIH mouse IFN reference units per mg protein) was produced and purified as previously described (199). IFN doses will be given in NIH mouse IFN reference units/ml (U/ml).

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Viruses. SV40 wild type strain KD was propagated in CV-1 cells by infection at an MOI of 0.01. When 90% of the cells showed cytopathic effects, the cells and the medium were frozen and thawed three times to lyse the cells and liberate the virus. The viral suspension was kept at -20°C. SV40 dl1263 was a gift from M.Polvino-Bodnar and C. Cole (Yale University) and was propagated as described above for wild type virus. SV40 rescued from SV40-transformed mouse cell line SV101 was donated by S. Chen (Columbia University).

Cells. African green monkey kidney cells CV-1 (ATCC # CCL70) and Vero (ATCC# CCL81) and SV40-transformed human cell line SV80 were obtained from the American Type Culture Collection, Rockville Md. SV40-transformed mouse cells SV3T3-38 and SV101 were gifts from P.W.J. Rigby and S.Chen, respectively. All cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Lab., Grand Island, N.Y.) supplemented with 10% fetal calf serum (GIBCO) in 5% CO_2 at 37°C.

SV40 infections. All viral infections were performed in minimal essential medium (MEM)(GIBCO) supplemented with 2% gamma globulin-free calf serum heat inactivated at 68°C for 60 min to eliminate antibody and complement reactions. SV40 was adsorbed to cells in one-tenth the volume of medium used for growth. CV-1 and Vero cells were infected at an MOI of 20 to 30, SV80 cells at an MOI of 100 to 150 and SV3T3-38 cells at an MOI of 200-250. SV40 titers were determined in CV-1P cells , a subclone of CV-1 cells, using agar as an overlay (200). The titers of the SV40 stocks were usually about 109 PFU/ml.

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Immunoprecipitation of Tag. Cells, treated with IFN and infected with SV40 if indicated, were grown in methionine-free minimal essential medium (GIBCO) supplemented with 5% calf serum (GIBCO) (which had been dialyzed against phosphate-buffered saline to reduce the methionine concentration) containing 50 to 100 µCi of [35S]methionine (Amersham Corp.) per 106 cells . After the indicated time the cells were washed three times with cold phosphate-buffered saline supplemented with aprotinin to 1%(w/v) and lysed with 200 µl of chilled 0.02M Tris-HCl (pH 8.0), 0.15M NaCl, 1% (w/v) Nonidet P-40 and 1mM phenylmethylsulfonylfluoride (PMSF) per 106 cells. Lysates were incubated at 4°C for 10 mins, collected in 1.5 ml Eppendorf tubes and either stored at -60°C or processed as follows. The lysates were centrifuged at 12,000 x g at 4°C for 10 min, and 5µl aliquots of the supernatant fractions were precipitated with chilled 10% (w/v) trichloroacetic acid (TCA) and counted. All further procedures with the supernatant fractions were performed using the same number of TCA precipitable counts. The fractions were precleared with 25ul of a Staphylococcus A IgSorb suspension (The Enzyme Center, Boston, Ma.) in order to reduce non-specific immunoprecipitation. The volume of each sample was adjusted to 200ul with NET buffer (0.15M NaCl, 5mM EDTA, 50mM Tris-HCl (pH 7.4), 0.03% Sodium Azide, 0.2%(w/v) gelatin, 0.05%(w/v) Nonidet P-40, 1mM PMSF) and the samples were supplemented with 25 µl of IgSorb and incubated at 4°C for 1h. The samples were centrifuged at 12,000x g at 4°C for 10 min. Each of the resulting supernatant fractions was supplemented with 25 µl of anti-Tag antiserum(provided by J Li, Becton Dickson and Co.,

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Paramus, N.J.) incubated at 4°C for 24 to 28 h, supplemented with 50μl of IgSorb and incubated further at 4°C for 1h. The samples were centrifuged at 12,000 x g at 4°C for 5 min and the pellet fractions were washed five times . Each wash consisted of suspension of the pellet fraction in 300μl of NET buffer with sonication and pelleting by centrifugation. After the last wash the pellet fraction was suspended in 0.1 ml of SDS-PAGE sample loading buffer containing 0.5mM dithiothreitol, boiled for 5 min, and centrifuged as above; 100μl of each supernatant fraction was analyzed by electrophoresis in polyacrylamide gels in the presence of SDS (201). The gels were fixed in 20% acetic acid-25% ethanol in water, fluorographed (using 22%(w/v) 2,5-diphenyloxazole in dimethylsulfoxide), dried, and visualized by autoradiography , using XAR-7 film (Eastman Kodak Co., Rochester, N.Y.) with one intensifying screen (Dupont).

RNA isolation and primer extension. Cells were harvested , washed extensively with Saline A (GIBCO), resuspended in RSB buffer (10mM Tris-HCl (pH 7.5), 10mM KCl, 3mM $MgCl_2$) and lysed by addition of Nonidet P-40 to 0.5% (w/v). Cytoplasmic extracts were made, treated with proteinase K and extracted with phenol:chloroform:isoamyl alcohol (50:49:1) as described in Ghosh et al (202). Polyadenylic acid containing RNAs were isolated and SV40 specific transcripts identified by the method of primer extension as described by Ghosh et al (202).

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RESULTS

IFN action: inhibition of SV40 Tag and early mRNA expression in CV-1 cells infected with SV40. We exposed monkey CV-1 cells to human alpha IFNs and found that the cells were responsive. Treating CV-1 cells with 100U/ml of of human alpha IFNs for 18 h before infection with VSV (at an MOI of 0.1) resulted in a decrease in the virus yield of about 1,000-fold (data not shown). Furthermore, CV-1 cells which had been exposed to 1,000 U/ml of human alpha-2 IFN for 18 h were infected with SV40 at an MOI of 20 and further incubated in the presence of IFN. At 96 h postinfection the IFN treated cells were completely protected from the viral cytopathic effects, whereas over 90% of the untreated cells showed cytopathic effects (data not shown).

We tested for the effect of IFN on Tag accumulation by incubating infected cells with [35S]-methionine and immunoprecipitating the Tag from cell lysates as described in the materials and methods. Treatment of CV-1 cells with human alpha IFNs for 24 h before infection with SV40 inhibited the accumulation of viral Tag (synthesized between 20 and 26 h post infection). The extent of the inhibition increased with the concentration of IFNs used, (Fig. 1). Inhibition was detectable at a concentration of 10 U/ml, more pronounced at a concentration of 100 U/ml and reached the maximal level at concentration of 1,000 U/ml, (Fig. 1), about 95% inhibition as detected by scanning the autoradiogram.

CV-1 cells were exposed to 1,000 U/ml of IFNs per ml for

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Figure 1. Inhibition of Tag expression in CV-1 cells exposed to IFNs before infection with SV40. CV-1 cells were exposed to different concentrations of human alpha IFNs for 24 h and infected with SV40 (moi=20). The cells were grown in the presence of [^{35}S]methionine from 20 to 26 h postinfection. Labeled Tag was immunoprecipitated, fractionated by SDS-PAGE and visualized by autoradiography. Tag (**a**), from infected, untreated cells (lane 1), and from infected cells treated with IFNs at concentrations of 10U/ml (lane 2), 100U/ml (lane 3), 1,000U/ml (lane 4), and 10,000U/ml (lane 5).

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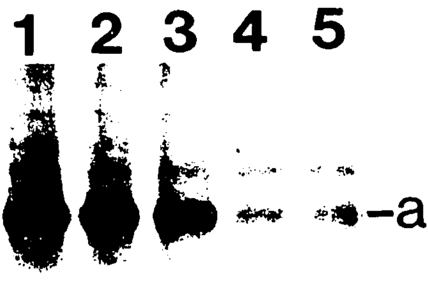

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24 h, infected with SV40, and further incubated in the presence of IFN for up to 100 h. The inhibition of Tag expression persisted throughout the 100 h (Fig. 2,lanes 14 and 15). However, 5000 U/ml of human alpha IFNs added 24 h after infection had no effect on the amount of Tag synthesized between 36 and 48 h after infection (data not shown).

We tested the effect of IFN treatment on SV40 mRNA accumulation by primer extension. A labeled oligodeoxynucleotide primer complementary to a segment of early SV40 mRNA was hybridized to polyadenylic acid-containing RNA isolated from CV-1 cells, extended with reverse transcriptase, fractionated by gel electrophoresis, and visualized by autoradiography. SV40-specific mRNA accumulation was inhibited in CV-1 cells which had been exposed to IFNs for 18 h before infection, (Fig. 3, lanes 2 and 3). In cells exposed to IFNs for 18 h before and after infection the inhibition of SV40 mRNA accumulation persisted for as long as it was tested (ie, 36 h postinfection) (data not shown).

Selectivity of IFN action in SV40-transformed cells infected with SV40. (i) Studies with the human cell line SV80. SV40 transformed SV80 human fibroblasts synthesize SV40 early RNAs and SV40 Tag. Treatment of SV80 cells with 1,000 U/ml of human alpha IFNs had little or no effect on the level of Tag expression. (Fig 4, lanes 1 and 2). This treatment conferred protection to the SV80 cells from the cytopathic effects of VSV

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Figure 2. Persistent inhibition of Tag expression in CV-1 cells continuosly exposed to IFNs and infected with SV40. CV-1 cells were treated with 1000U/ml of human alpha IFNs for 24 h, and infected with SV40 (moi=20). At 1h postinfection IFNs were readded at a concentration of 1000U/ml. The medium and the IFNs were replaced every 24 h. Immunoprecipitated Tag was visualized as in Fig. 1, except that labeling with [^{35}S]methionine was for 12 h prior to extraction. Tag (**a**) from IFN treated SV40 infected cells (odd lanes), SV40 infected cells (even lanes), IFN treated, uninfected cells (lane 15), and uninfected cells (lane 16) is shown. Protein was extracted at 28 h (lanes 1,2,15, & 16), 40 h (lanes 3 & 4), 52 h (lanes 5 & 6), 64 h (lanes 7 & 8), 76 h (lanes 9 & 10), 88 h (lanes 11 & 12), and 100 h post infection (lanes 13 & 14).

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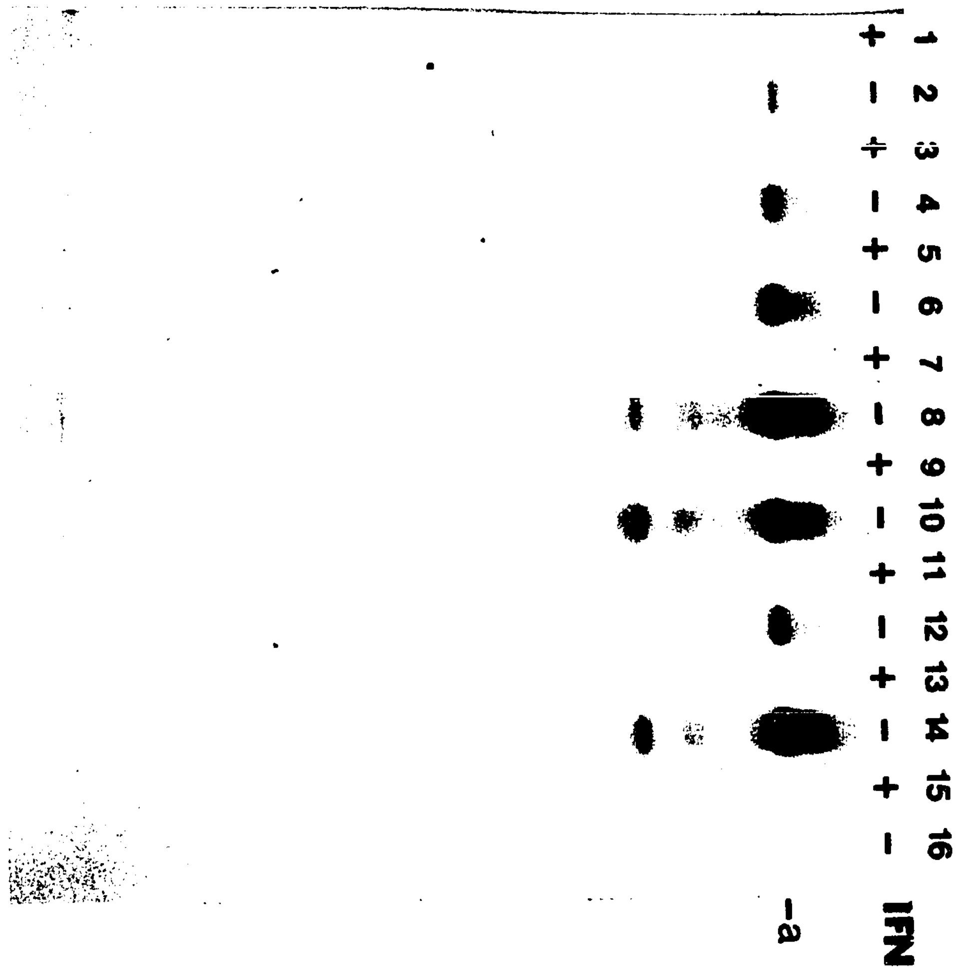

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Figure 3. Inhibition of the accumulation of SV40 early RNAs in CV-1 cells treated with IFNs and infected with SV40. CV-1 cells were treated with 500U/ml of human alpha IFNs for 18 h and infected with SV40. Cytoplasmic RNA was extracted at 22 h postinfection. PolyA(+) RNA was prepared and used as template for primer extension by reverse transcriptase. The primer used spanned nucleotides 5,055 to 5,091 of the SV40 genome. The extension products were fractionated in sequencing gels and were visualized by autoradiography. Shown here are extension products of: RNA extracted from SV80 cells (lane 1), infected CV-1 cells (lane 2), and IFN treated infected CV-1 cells (lane 3).

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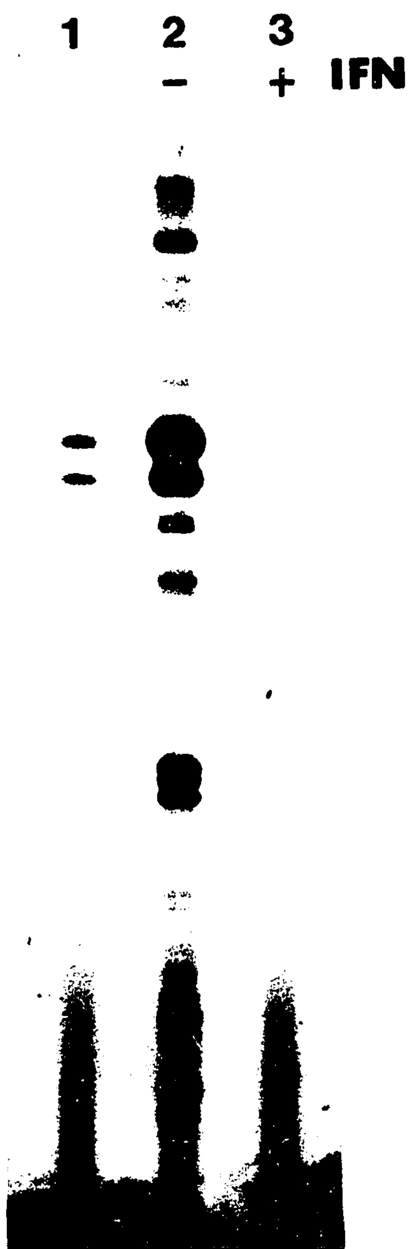

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SV40 dl1263 is a viable SV40 deletion mutant which specifies a truncated Tag (203). SV80 cells infected with SV40 dl1263 synthesize two types of Tag, the 88,000-dalton endogenous Tag specified by the integrated SV40 genome and the 83,000-dalton Tag specified by the infecting SV40 dl1263 virus. When SV80 cells were treated with 1,000 U/ml of human alpha IFNs and infected with SV40 dl1263, accumulation of the SV40 dl1263-specific 83,000-dalton Tag was almost completely abolished, whereas accumulation of the endogenous 88,000-dalton Tag was not significantly affected, (Fig. 4, lanes 3 and 4). IFN treatment up to 10,000 U/ml did not affect the expression of the endogenous Tag; the exogenous Tag expression was reduced by 50% with 100 U/ml and was completely inhibited with 10,000 U/ml. (data not shown).

The time of IFN treatment was important; all the experiments above were done by adding IFN to the cells for 18 to 24 h before infection and continuing the IFN treatment after infection. However, if 1,000 U/ml of IFN was added at the time of infection the inhibition of the exogenous Tag was only 50% , if added 15 h post infection there was no inhibition (data not shown).

(ii) Studies with the mouse cell line SV3T3-38. SV40- transformed SV3T3-38 mouse fibroblasts have SV40 DNA integrated into the cellular DNA. This integrated endogenous SV40 genome has been cloned and its restriction enzyme cleavage pattern has been found to correspond to that of SV40 in the region coding for

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Figure 4. Selectivity of IFN action: inhibition of the expression of the Tag specified by the infecting viral genome in SV40 transformed SV80 cells infected with SV40. SV80 cells were treated with 1000U/ml of human alpha IFN for 18 h and infected with SV40 deletion mutant dl1263, which encodes a truncated Tag. IFNs were re-added 1 h postinfection. The SV80 Tag (**a**) had an apparent molecular weight of 88,000 daltons, and the dl1263 Tag (**b**) had an apparent molecular weight of 83,000 daltons. Tag was analyzed as in Fig. 1, except that protein was extracted at 44 h postinfection. Tag was immunoprecipitated from uninfected SV80 cells (lane 1), IFN treated SV80 cells (lane 2), dl1263 infected SV80 cells (lane 3), and IFN treated, dl1263 infected SV80 cells (lane 4). The protein size markers used were ovalbumin (43 kD), bovine serum albumin (66.2 kD), and phosphorylase b (92.5 kD).

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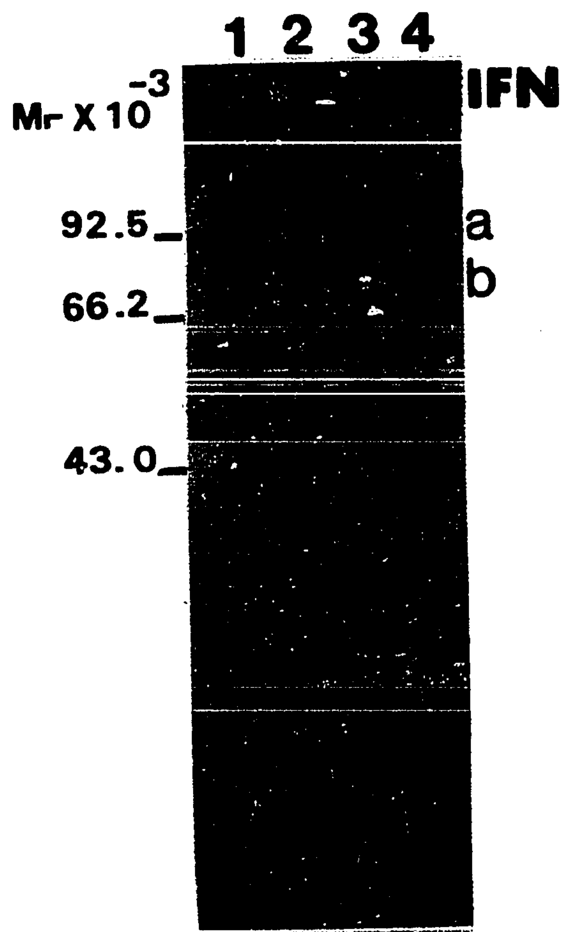

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Figure 5. Selectivity of IFN action: inhibition of the expression of the Tag specified by the infecting viral genome in SV40 transformed SV3T3-38 cells infected with SV40. SV3T3-38 mouse cells were treated with mouse ß IFN for 18 h and infected with SV40 dl1263. IFN was re-added 1 h postinfection, and Tag was visualized as in Fig. 4. **A.** The immunoprecipitated SV3T3-38 Tag (**a**), with an apparent molecular weight of 88,000 daltons, and the dl1263 Tag (**b**) were visualized as in fig. 1, and are shown for uninfected SV3T3-38 cells (lane 1), SV3T3-38 cells treated with 10,000U/ml of ß IFN (lane 2), dl1263 infected SV3T3-38 cells (lane 3), dl1263 infected SV3T3-38 cells treated with 1,000U/ml of ß IFN (lane 4), and dl1263 infected SV3T3-38 cells treated with 10,000U/ml of ß IFN (lane 5). The protein size markers used were as in Fig. 4. **B.** Portion of the same gel, enlarged to improve the resolution of **a** and **b**.

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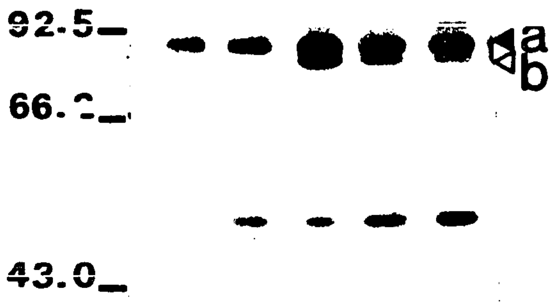
A 1 2 3 4 5

Mr X 10^{-3} − + − + + IFN



B 1 2 3 4 5

− + − + + IFN

a

b

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Tag. The integration occurred in the late region of the SV40 genome (204).

SV3T3-38 cells synthesize SV40 specific RNAs and Tag. Treatment of SV3T3-38 cells with 10,000 U/ml of mouse ß IFN had little or no effect on the level of the Tag, (Fig. 5, lanes 1 and 2). This line was shown to be protected against VSV by IFN (data not shown).

SV3T3-38 cells infected with SV40 dl1263, synthesize two types of Tag, the 88,000-dalton endogenous Tag specified by the integrated SV40 genome and the 83,000-dalton Tag specified by the infecting virus. When these cells were treated with 1,000 U/ml or 10,000 U/ml of pure mouse ß IFN and infected with SV40 dl1263 accumulation of the SV40 specific 83,000-dalton Tag was almost completely inhibited, whereas accumulation of the 88,000-dalton Tag was not significantly affected, (Fig. 5, lanes 3 through 5). 100 U/ml of the mouse ß IFN reduced the expression of the exogenous Tag by 50%. As in the case of the SV80 cells IFN had to be added prior to infection to have this effect (data not shown).

Selectivity of IFN action: inhibition of expression of Tag of SV40 rescued from the SV40-transformed mouse cell SV101. We wanted to determine whether the lack of effect of IFN on the expression of Tag specified by an integrated SV40 genome was a consequence of an irreversible alteration of the SV40 sequence. To do this we used SV40 which was rescued from the SV40-transformed cell, SV101, by fusing these cells with permissive CV-1 cells .

The level of Tag expression in SV101 cells was only slightly diminished in the cells exposed to 1,000 U/ml of mouse ß

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IFN for 24 h before infection, (Fig. 6, lanes 1 and 2). Infection of Vero cells with the SV40 rescued from SV101 cells resulted in the synthesis of a series of Tags (Fig 6, lane 5). Theses probably arise from excisional recombination (204b). In Vero cells which had been treated with 1,000 U/ml of human alpha IFNs before infection with the rescued virus, the expression of Tag was almost completely inhibited (Fig. 6, lanes 3 through 6). This indicates that the expression of the Tag specified by rescued virus is as sensitive to IFN as Tag expression specified by wild type virus.

DISCUSSION

The results of our experiments with CV-1 cells confirm and extend the results of previous studies from other laboratories (see review in chapter one). The data indicate that human IFNs added before SV40 infection inhibit the accumulation of SV40 early mRNA and Tag. In the constant presence of IFNs this inhibition persists for as long as the preparations were tested. Therefore, the inhibition of early SV40 mRNA and Tag accumulation could account for the inhibition of viral growth seen after IFN treatment and does not represent only a delay in the appearance of the early products of SV40.

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Figure 6. Selectivity of IFN action: inhibition of the expression of Tag in cells infected with SV40 rescued from SV40 transformed cells. SV101 cells were treated with 1,000U/ml of mouse ß IFN for 18 h. Tag (**a**) was visualized as in Fig. 1, and is shown for SV101 cells (lane 1) and IFN treated SV101 cells (lane 2). Vero cells were treated with 1000U/ml of human alpha IFNs for 18 h and infected with SV40 rescued from SV101 cells. Protein was extracted at 32 h postinfection, Tag was visualized as in Fig. 1, and is shown for uninfected Vero cells (lane 3), IFN treated Vero cells (lane 4), Vero cells infected with rescued virus (lane 5), and IFN treated Vero cells infected with rescued virus (lane 6). The SV40 rescued from SV101 specified Tags (**a**) of at least three sizes.

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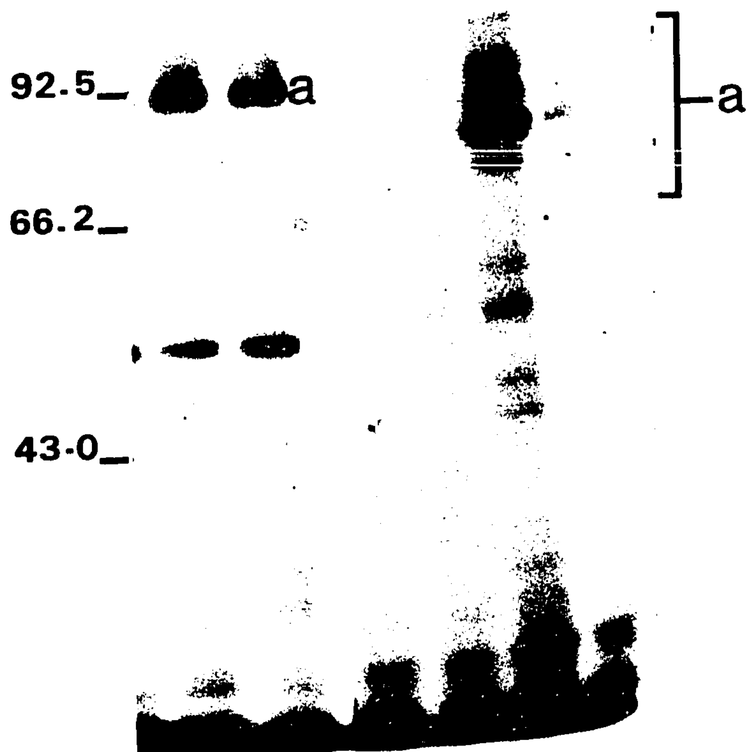

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Experiments with SV40-transformed human SV80 and mouse SV3T3-38 cells revealed that even in the same cell IFNs inhibit the expression of Tag encoded by infecting SV40 but have almost no effect on the expression of Tag encoded by an integrated SV40 genome. This was established by using an SV40 mutant, SV40 dl1263, which encodes a truncated Tag, smaller than the Tag encoded by the SV40 DNA integrated in SV80 and SV3T3-38 cells. This mutant behaves like the wild type virus in lytic infections of monkey cells (203). This demonstration of the selectivity of IFN action provides direct evidence for previous assumptions (156,169,175,182) and eliminates the possibility that SV40-transformed cells could be nonresponsive to IFNs with respect to SV40 infection (76).

The mRNAs coding for Tag in cell line SV80 have the same 5' and 3' ends as SV40 mRNAs in early lytic infections (205,206, and fig. 3 lane 1). Thus , the lack of a pronounced effect of IFN on the accumulation of the Tag specified by the integrated SV40 DNA in these cells cannot be due to host sequences joined at the 5' or 3' ends of the SV40 mRNAs. The very unlikely possibility that there are host sequences joined to the nuclear precursors of SV40 mRNAs was not eliminated in the case of this cell line (206).

No difference was detected between the restriction maps of the gene coding and flanking regions in the integrated SV40 DNA in SV3T3-38 cells when compared to wild type SV40 (204). Thus the lack of inhibition of Tag expression in this cell line when treated with IFN is unlikely to be a consequence of the replacement of the

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SV40 promoter by a host promoter or the insertion of host sequences in the mRNAs.

Tag expression in SV101 cells was insensitive to IFN treatment, whereas Tag expression during lytic infection of Vero cells with the SV40 rescued from SV101 cells was inhibited by IFN treatment. These observations indicate that integration into the host chromosome does not result in deletion of sequences which make SV40 sensitive to IFN action.

The inhibition of the early SV40 RNAs could involve decreased synthesis. It is probable that the transcription from a newly introduced gene and a stably integrated gene would be controlled by different factors, as was shown for the E1A activation of the rat preproinsulin gene (207). IFNs may block the formation of an active transcription complex after SV40 DNA enters the nucleus. On the other hand IFN treatment may cause a faster turnover of SV40 RNAs, (a probable mechanism in the case of reovirus infection of IFN-treated cells, (208)); the selectivity could be provided by a localized activation of the RNase L as was postulated by Baglioni (100).

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CHAPTER THREE

EFFECT OF INTERFERONS ON TRANSFECTED GENES.

INTRODUCTION.

The inhibitory effect of IFN on the expression of SV40 early transcripts might be a consequence of one of the following: 1) inhibition of viral adsorption and/or entry into cells and nuclei, 2) inhibition of viral uncoating; 3) inhibition of transcription , 4) degradation of SV40 RNAs, and 5) inhibition of translation.

Oxman and coworkers and Yamamoto et al. showed that IFN treatment had no effect on viral adsorption or on viral entry into cells and nuclei (152,156). Furthermore, Oxman et al found that IFN treatment does not alter the time needed for, or the final equilibrium of, the conversion of the SV40 DNA from a DNase resistant to a DNase sensitive form after infection of cells (152). The authors concluded that IFN did not inhibit uncoating of the virions (152).

Other groups studied uncoating using an indirect method: SV40 DNA transfection. Yamamoto et al. showed that pre-treatment with IFN only weakly inhibits viral DNA synthesis and

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expression of Tag when SV40 DNA is transfected into cells (156). Brennan and Stark reported that IFN did not decrease SV40 RNA accumulation when SV40 DNA is transfected into CV-1 cells (169). However, work of Mozes and Defendi contradicts this, showing that IFN treatment inhibits the accumulation of SV40 RNAs and Tag when SV40 DNA was transfected into cells (168). Revel reviewing unpublished data from his laboratory agreed with the latter (160).

We decided to study the effect of IFN on the expression of newly introduced genes. Here I show that 1) IFN inhibited the expression of SV40 early products without affecting viral entry into cells, and 2) IFN inhibited the replication and expression of viral products when SV40 DNA was transfected into cells. Furthermore, 3) IFN inhibited the expression of other genes, including a non-viral gene, when these genes were transfected into cells.

MATERIALS AND METHODS

DNA. SV40 form I DNA was made from virally infected cells following the procedure of Hirt. (209) pPSV2gpt, containing the E. coli xanthine-guanine phosphoribosyltransferase gene driven by the SV40 early promoter was obtained from Dr. H. Schmidt, pSV2CAT was the gift of Dr. E. Feingold and pabCAT was a gift of Drs. H. Samanta and D. Engel (52).

Ifns. As described in chapter two.

Virus. SV40 (wt) KD strain was grown as described in chapter two, and purified by pelleting cell debris at 10,000 G at 4°C for 1h, respining the supernatant at 80,000 G at 4°C for 2h . The

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pellet fraction was resuspended and layered on a CsCl solution (0.5g/ml) followed by spinning at 80,000 G at 15°C for 48h. The visible SV40 band was collected and dialysed free of CsCl.

Cells. As described in chapter 2 except the following: COS-1 cells, a derivative of CV-1 cells stably transformed with an ori-mutant of SV40, were a gift of Dr. P.K. Ghosh and were grown as described for CV-1 cells. HGPRT- chinese hamster V-79HP (V-79) cells were a gift of Dr. H. Schmidt and were grown as described by Yoshie et al. (56). Hela cells were grown as monolayers in DMEM supplemented with 10% fetal calf serum.

Infections and Transfections. Infections with SV40 were as described in chapter two. Transfections were done following a modification (210) of the method of Van der Eb et al (170). Usually 1 µg of plasmid DNA was transfected with sonicated salmon sperm DNA as carrier (9-15 µg) per 10^6 cells. Selection of gpt+ cells involved growth in HAT medium (medium supplemented with 13.3µg/ml hypoxanthine, 0.88µg/ml aminopterin, 3.6µg/ml thymidine) (211), colonies were counted three weeks after transfection.

CAT assays. CAT assays were as described by Gorman et al. (212), with the reaction products chromatographed on silica thin layer plates (Analtech) with chloroform:methanoi (95:5). (52,212)

Southern and northern blot analysis. DNA was isolated by the method of Hirt (209) and analyzed by Southern analysis as described in (213). RNA was isolated as described in chapter 2 and analyzed by northern blot analysis (213). The SV40 specific probe was the Hind III-A fragment of SV40 DNA, nick-translated following

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the procedure of Rigby et al. (214).

RESULTS

Effect of interferon on replication and expression of SV40 after transfection of SV40 DNA into monkey cells. We labeled SV40 in vivo with [35S]methionine or [32P]Pi, purified the virions as described above and infected Vero cells with labeled virus. At various times after infection, cells were washed thoroughly with PBS, and lysed as described in the previous chapter. The nuclei were washed by serial spins in the presence of NP40; the radioactivity in the nuclear and cytoplasmic fractions was counted in a scintillation counter. We found that treatment with doses of IFN which inhibited the expression of Tag, did not inhibit entry of the labeled virus into cells and nuclei (data not shown).

Using the same viral preparations we attempted to study viral uncoating following the procedure of Barbanti-Brodano et al (215). This method was reported to measure a change in density of the viral particles after infection as a direct measurement of uncoating. We could never reproduce Barbanti-Brodano's methodology (others have reported the same difficulty (156)). Therefore, we decided to investigate the question of uncoating indirectly by studying the effect of IFN treatment on SV40 functions following SV40 DNA transfection into monkey cells.

CV-1 cells were treated with 5000 U/ml of human alpha IFNs for 18 h and then transfected with SV40 DNA or infected with SV40 virions. IFN treatment inhibited the expression of Tag as detected by immunoprecipitation ; in both infected and transfected

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cells (data not shown).

CV-1 cells were treated with 1000U/ml of human alpha IFN for 18 h and transfected with increasing amounts of SV40 DNA. Total cytoplasmic RNA was isolated 36 h post transfection and was fractionated by electrophoresis. Northern blot analysis revealed that there was an almost complete inhibition of the expression of these RNAs in transfected cells that had been treated with IFN (Fig 7A, compare untreated cells, lanes 2,4,6 & 8; with IFN treated cells, lanes 3,5,7 & 9). When the amount of transfected SV40 DNA was 3μg per 106 cells (lane 8) the IFN effect was diminished (lane 9).

CV-1 cells were treated with human alpha-2 IFN (1000U/ml) and transfected or infected with SV40 DNA, 36 h post-transfection SV40 DNA was extracted by the method of Hirt (209), digested with Hind III, fractionated by electrophoresis and analyzed on a Southern blot. IFN treatment decreased the level of SV40 DNA present in infected and transfected cells. The inhibition of SV40 DNA accumulation after infection was close to four fold (not shown), whereas the inhibition after transfection was three fold when using 1μg of DNA per 106 cells (Fig. 7B, lanes 1 & 3) and ten fold when using 0.1μg per 106 cells (lanes 2 & 4)

Effect of interferons on transfection efficiency of V79 cells with pSV2gpt. V-79 cells were treated with varying doses of mouse ß IFN for 24 h prior to the transfection and for 24 h post-transfection. These cells, which are HGPRT-, were transfected with pSV2gpt, which encodes a XGPRT enzymatic activity. Starting 48 h post transfection, the cells were grown in

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Figure 7. Effect of IFN on replication and expression of SV40 after transfection of SV40 DNA into monkey cells. **A.** CV-1 cells were treated with 1000U/ml of human alpha IFNs for 18 h and transfected with SV40 DNA. RNA extracted at 36 h post-transfection, was fractionated in formaldehyde-formamide agarose gels, transferred to nitrocellulose filters and hybridized to nick-translated Hind IIIA fragment of the SV40 genome. SV40 specific RNA was visualized by autoradiography and shown for mock-transfected cells (lane 1), cells (**c**)transfected with 0.05μg, 0.1μg, 0.5μg, and 3.0μg of SV40 DNA per 10^6 cells (lanes 2, 4, 6, & 8 respectively), and IFN treated cells (**i**) transfected with 0.05μg, 0.1μg, 0.5μg, and 3.0μg of SV40 DNA per 10^6 cells (lanes 3, 5, 7, & 9,). **B.** CV-1 cells were treated with 1000U/ml human alpha-2 IFN and transfected with SV40 DNA. DNA extracted 36 h post-transfection was digested with Hind III, fractionated in an agarose gel, transferred to nitrocellulose filters and hybridized to the same probe as in A. The level of SV40 DNA was determined by visualizing the hybridization to the Hind III A fragment and this is shown for CV-1 cells transfected with 1μg or 0.1μg of SV40 DNA per 10^6 cells (lanes 1 & 2 respectively) and for IFN treated cells transfected with 1μg or 0.1μg of SV40 DNA per 10^6 cells (lanes 3 & 4 respectively).

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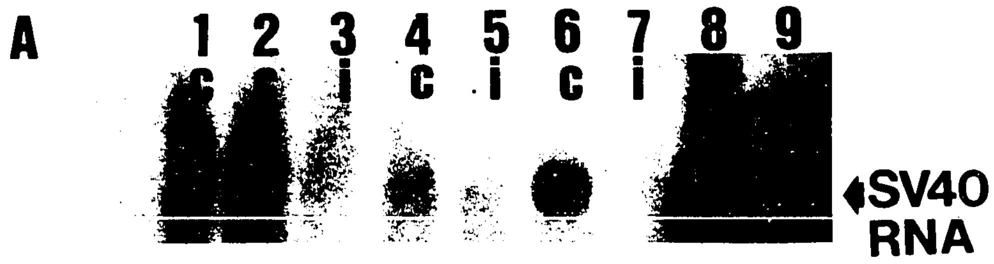

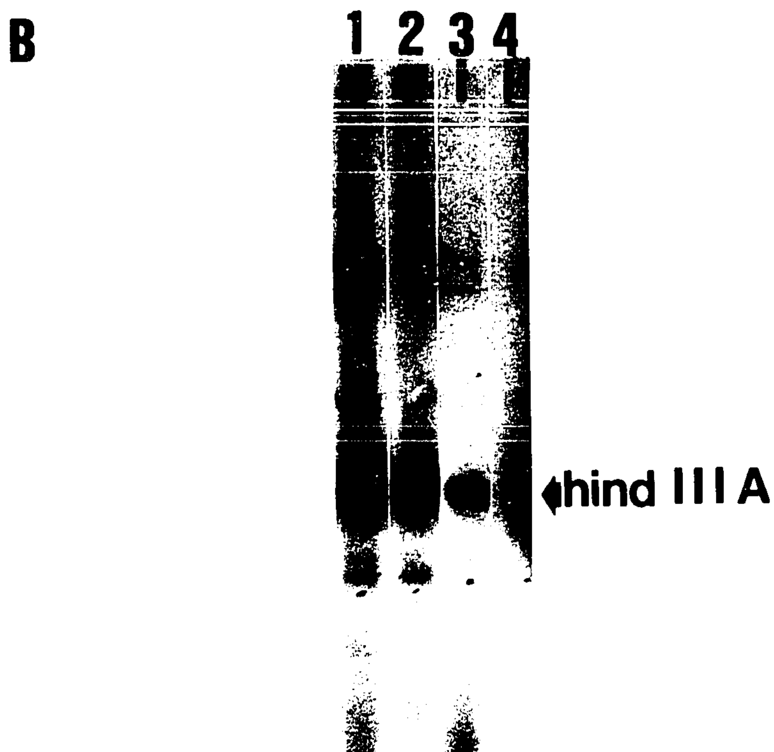

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HAT medium and resistant (XGPRT+) colonies were counted three weeks later. IFN treatment resulted in a profound decrease in the number of resistant colonies obtained (Fig. 8A). 50U/ml of IFN was sufficient to inhibit colony formation by more than 50% (Fig. 8A), the dose response was very similar to that noted for the inhibition of Tag expression after SV40 infection. (see Fig. 1)

In order to determine if the time of treatment with IFN was important, we treated V-79 cells with 500 U/ml of mouse ß IFN for different lengths of time (Fig. 8B), and performed the same experimental procedure as described above. Treatment with IFN starting after the transfection did not inhibit transformation (Fig. 8B). Pretreatment for 12 h before transfection seemed to yield maximal inhibition, Fig. 8B.

In two separate experiments, we observed inhibition (77% and 88%) of colony formation if 2 ug of (2'-5')pppApApA was co-transfected with pSV2gpt (not shown).

We performed several experiments to evaluate the role of growth inhibition by IFN in the above results. The growth of V-79 cells was not inhibited by 500 U/ml ß IFN if the treatment lasted less than 36 h (not shown). pSV2gpt transformed V-79 cells (XGPRT+) were plated in HAT medium so as to recover individual clones (a plating efficiency assay). 500 U/ml of ß IFN, added upon seeding the plates and then every 48 h for two weeks, had only a small effect on the number of colonies obtained: 617 for the untreated, versus 528 colonies for the treated cells (not shown). The colonies from IFN treated cells were smaller.

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Figure 8. IFN dose and time dependent inhibition of transfection by pSV2gpt. **A.** V79 cells were treated with mouse ß IFN for 24 h, transfected with 1μg of pSV2gpt per 10^6 cells using the method of Van der Eb (170). IFN was re-added 1 h after transfection. Starting 24 h after transfection the cells were grown in HAT medium in the absence of IFN. The medium was exchanged for fresh HAT medium every 24 h and surviving colonies of cells were counted two weeks post-transfection. The results are given in % of control: cells not treated with IFN. **B.** V79 cells were treated with 500u/ml of mouse ß1 IFN for varying lengths of time, cells were trransfected and HAT resistant colonies obtained as in A. The results are given in % of control. Time before transfection (-), time after transfection (+), no SV40 DNA (-DNA).

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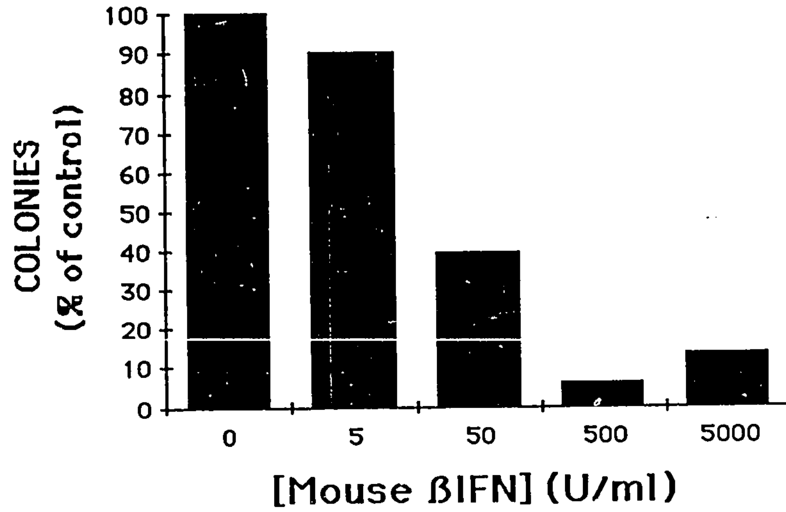
A



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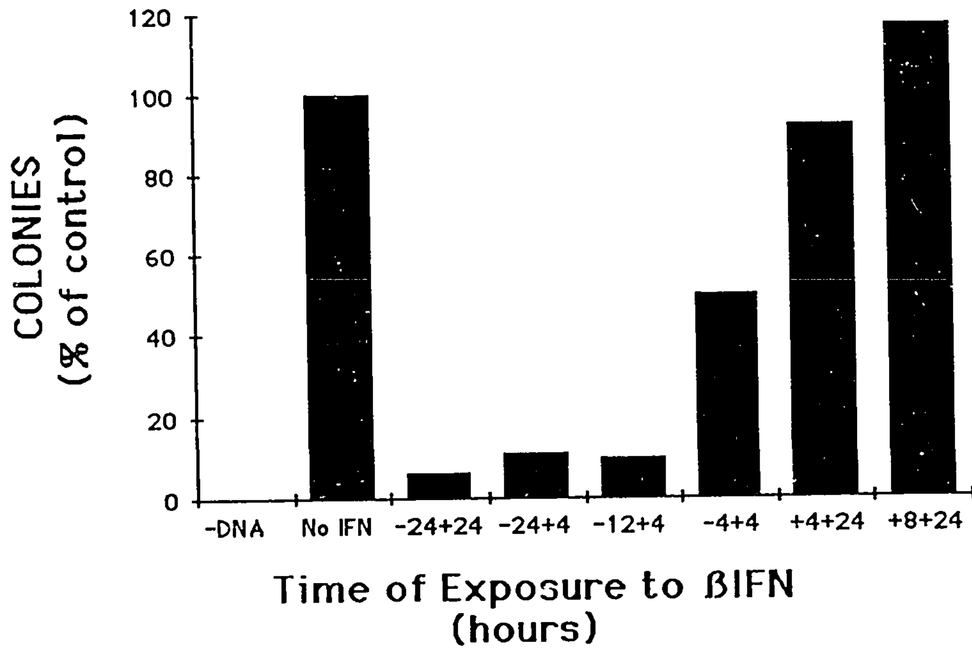

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[32P]-labeled pSV2gpt DNA was used to follow the fate of the transfected DNA . IFN treatment did not alter the level or the kinetics of entry of the DNA into the cells or of its disappearance from the Hirt supernatant fraction (data not shown).

Effect of IFN on the transient expression of transfected genes. We treated Hela cell monolayers with human alpha-2 IFN and transfected the cells with pSV2CAT. Cell lysates were made and CAT activity was assayed as described in Samanta et al (52). Pretreatment for 18 h before transfection, and continued presence of 1000U/ml of human alpha-2 IFN after transfection inhibited the CAT activity more than five fold (Fig. 9, lanes 1 & 2).

Hela cells were treated with human alpha-2 IFN (starting 18 h prior to transfection (Fig. 9, lane 2), coincident with transfection (lane 3), or 18 h (lane 4), 24 h (lane 5) or 36 h after transfection (lane 6)), transfected with pSV2CAT, and CAT assays performed with extracts made at 48 h post-transfection. It is clear that pre-treatment with IFN was required to obtain maximal inhibition of the expression of pSV2CAT (Fig. 9, lanes 1 & 2).

We transfected Hela cells with pSV2CAT or pabCAT. pabCAT is a plasmid which contains a promoterless CAT gene driven by a DNA segment containing the promoter and enhancer elements of an IFN inducible gene (gene 202) in the mouse (52, Gribaudo and Lengyel, unpublished data). Samanta et al have shown that stable transformants of pabCAT have increased CAT activity after IFN treatment (52). When Hela cells were treated with human alpha-2

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Figure 9. IFN mediated inhibition of the transient expression of the CAT gene after transfections with pSV2CAT. Hela cells were treated with 1000U/ml of human alpha-2 IFN for varying lengths of time and transfected with 1μg of pSV2CAT DNA per 10^6 cells as described in Fig. 8. extracts were made48 h post transfection. Chloramphenicol acetyltransferase (CAT) activity was assayed as described in Samanta et al (52). The TLC-fractionated derivatives of [^{14}C]chloramphenicol were visualized by autoradiography. This is shown for transfected HeLa cells (1), and for Hela cells treated with IFN starting at the following times: 18 h before transfection (2), coincident with transfection (3), 18 h post transfection (4), 24 h post transfection (5), 36 h post transfection (6). Duplicates or triplicates are from independent Hela cell cultures treated identically. Also shown are derivatives of [^{14}C]chloramphenicol when incubated with authentic CAT enzyme (E), and [^{14}C]chloramphenicol (CM). [^{14}C]chloramphenicol (**a**); contaminant in the CM preparation, (**b**); 1-acetylCM, (**c**); 3-acetylCM, (**d**); origin, (**o**). Each lane shows at least a pair of duplicate Hela cell cultures treated identically.

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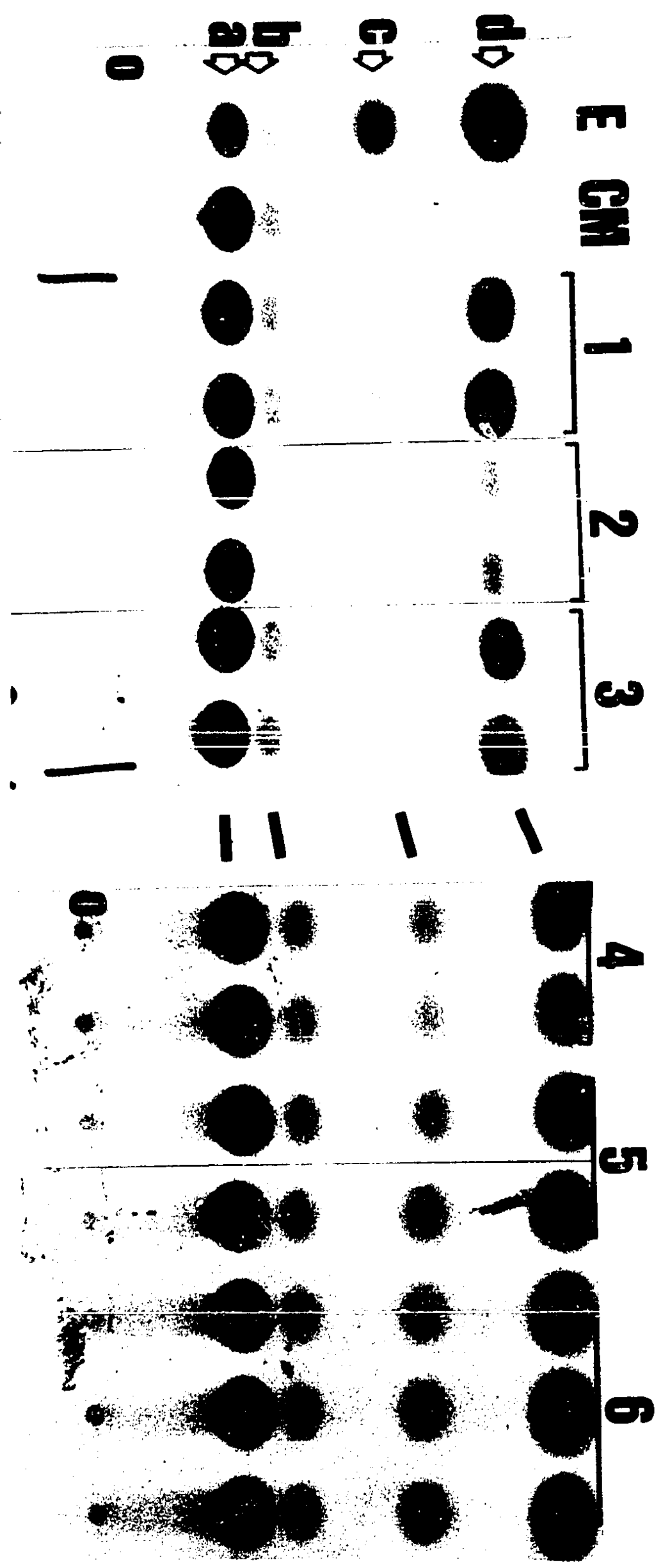

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IFN and then transfected with pSV_2CAT or pabCAT there was a marked inhibition of CAT activity compared to untreated cells with either of the plasmids used (Fig. 10A, compare lanes 2 &3 for pabCAT, and 5 & 6 for pSV2CAT; see also Fig. 10B). If the IFN was added after the transfection no significant effect was seen in transient assays (Fig. 10A, lanes 1 &4, see also Fig. 10B)(52).

DISCUSSION

IFN does not inhibit viral entry. Using labeled purified SV40 virions we found evidence that IFN does not affect entry of the virus into the cell nucleus. This agrees with previous studies of Oxman et al and Yamamoto et al (152,156). The only experiments dealing with the question of whether uncoating is blocked by IFN were done by Oxman (152), as described above. The conclusion from these is that IFN does not block the uncoating necessary to make the viral genome sensitive to DNase.

IFN inhibits SV40 specific functions after SV40 DNA transfection. As was stated above there is controversy in the literature as to whether IFN inhibits expression of SV40 gene products after transfection of SV40 DNA. The most careful study among the ones quoted is that of Mozes and Defendi; in which IFN was shown to inhibit SV40 expression whether the viral genome was introduced as part of infecting virions or as transfected DNA. Our results agree with this study, we show an inhibition of the SV40 DNA mediated SV40 early RNA expression and DNA accumulation by IFN (Fig. 7). Mozes and Defendi postulated that the lack of inhibition seen by Yamamoto et al could be explained if an excess of

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Figure 10. IFN mediated inhibition of CAT gene transient expression driven by viral or cellular promoter/enhancer elements. **A.** Hela cells were treated with 5000U/ml of human alpha-2 IFN either 24 h before transfection or 24 h post-transfection. The cells were transfected with 1μg of pSV2CAT or 1μg pabCAT (see ref. 52) per 10^6 cells. CAT activity in cells was determined as described in Fig. 9. The acetylated derivatives of [^{14}C]chloramphenicol were visualized by autoradiography after fractionation by TLC. These results are shown for: cells transfected with pabCAT and treated 24 h later with IFN (1), cells pretreated for 18 h h with IFN and transfected with pabCAT(2), cells transfected with pabCAT (3), cells transfected with pSV2CAT and treated 24 h later with IFN (4), cells pretreated with IFN and transfected with pSV2CAT (5), and cells transfected with pSV2CAT (6). [^{14}C]chloramphenicol (**a**); contaminant in the [^{14}C]chloramphenicol (CM) preparation (**b**); 1-acetylCM (**c**); 3-acetylCM (**d**); origin (**o**). Each lane shows a pair of duplicate Hela cell cultures treated identically. **B.** The average amount of 3-acetylCM for each of the conditions described in A was determined by quantification of the autoradiographic signal with a soft laser scanner. the results are presented as % of control:from cells not treated with IFN. 3-acetylCM levels in extracts from cells transfected with pabCAT (black bars) or with pSV2CAT (gray bars) are shown.

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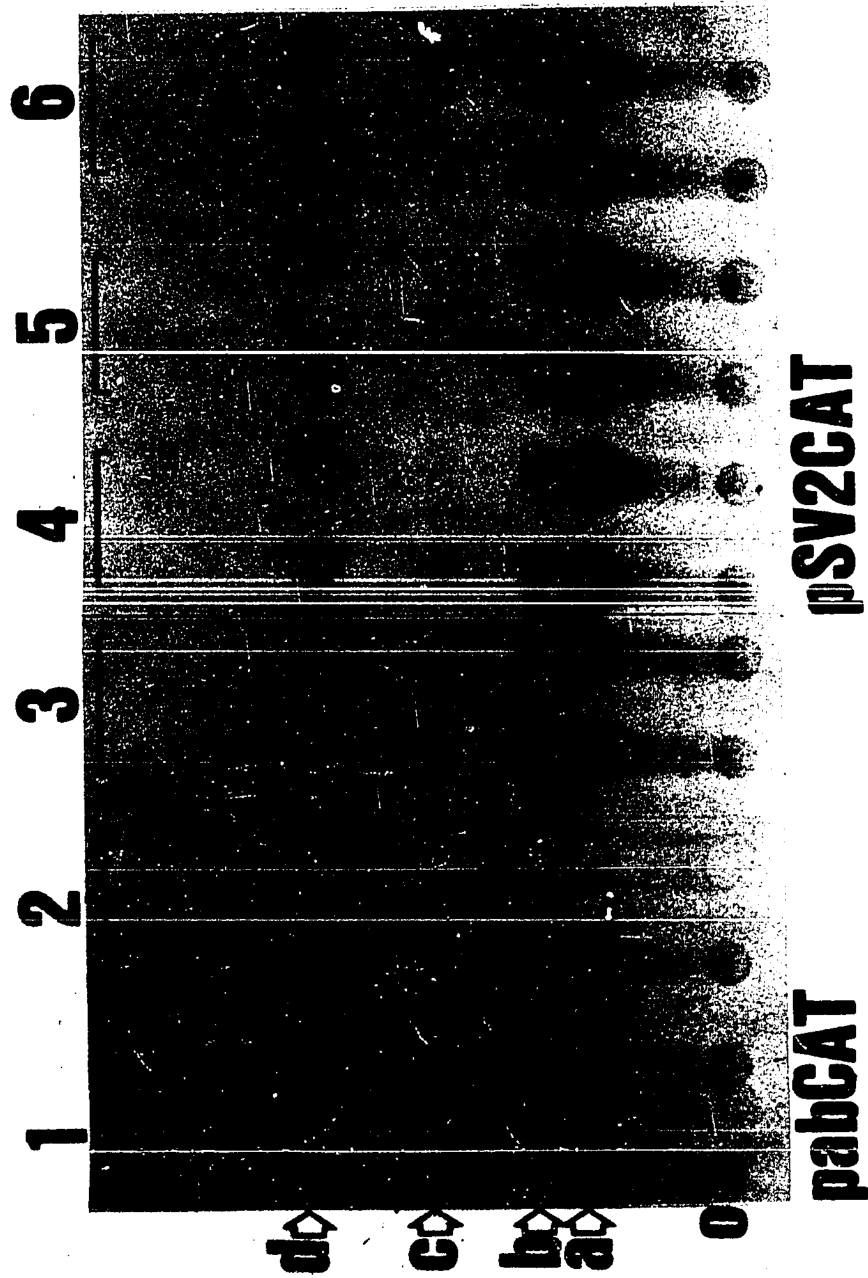

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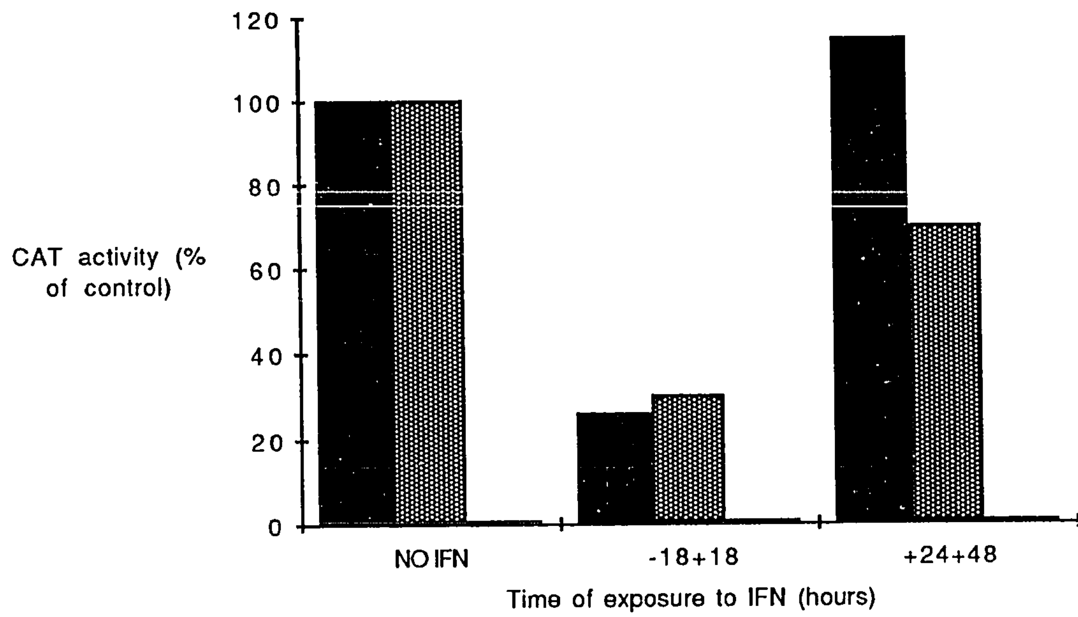

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SV40 DNA was used. This would override the IFN effect, much as the virus does (168). Our data on SV40 DNA dose response (Fig 7, lanes 8 & 9) support the idea that the effect of IFN is diminished at high SV40 DNA concentrations.

IFN inhibits the expression of transfected genes. We showed that IFN inhibited the transfection efficiency of pSV2gpt on V79 (HGPRT-) cells. This required IFN pretreatment (Fig. 8), similar to what we and others have described for the IFN mediated inhibition of SV40 infection (168). Cellular growth inhibition by IFN could not explain the results seen; furthermore plating efficiency of pSV2gpt transformed V79 (HGPRT+) in HAT medium was not significantly affected by the presence of IFN. Moreover, this demonstrated that the gpt gene, driven by the SV40 promoter-enhancer sequences, was insensitive to IFN treatment once this gene was integrated into the cellular genome of stable transfectants.

IFN did not affect DNA entry into the cells. Our favored interpretation of these experiments is that IFN inhibited the expression of the selectable marker gene and the cells died when switched to HAT medium.

M.-F. Dubois et al have published the following results: 1) When Ltk- cells were treated with mouse EAT cell IFN (0.1% pure) before transfection with the Herpes simplex virus tk gene, IFN treatment decreased the number of tk+ colonies obtained (216). 2) When these cells were transfected and then continually treated with IFN until colonies appeared, the transfection efficiency was also decreased by IFN , 3) furthermore other genes including cellular genes were equally inhibited (216). 4) Surprisingly, the authors

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claimed that IFN induced the expression of the transfected tk gene under the conditions above (216). Samid et al (217) and Perucho and Esteban (218) repeated some of these experiments and obtained similar results when IFN pretreatment was tried; in agreement with our results. The effect seen when IFN was added after transfection probably depends on the duration of the IFN exposure, we did not see a significant inhibition with short exposures, and never tried long ones.

IFN treatment inhibited the expression of CAT activity when IFN treatment preceeded the transfection with CAT expression vectors. This effect was seen whether the promoter-enhancer sequences driving CAT expression were SV40 sequences or mouse genomic sequences (Figs. 9 &10). The mouse genomic sequences driving the CAT gene in pabCAT contain an IFN inducible element, which clearly increases expression of CAT activity in IFN treated cells stably transformed with pabCAT (52). The inhibition of the expression of PSV2CAT and pabCAT is in direct contradiction to the conclusions of Dubois et al (216); however, we have not tested the HSV tk gene. In agreement with our data, Kerr et al have observed a general inhibition of transient expression of transfected genes in IFN pretreated cells (Ian Kerr, personal communication). It is possible that this effect may be partially mediated at the translational level. Expression of the adenovirus VA RNA1, an inhibitor of the dsRNA dependent kinase, can enhance transiation of transfected genes without affecting translation of genomic genes (218b, 218c). This may be the result of local accumulation of dsRNA.

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Again, IFN seems to selectively inhibit the expression of genes when newly introduced but not once integrated in the cellular genome (see chapter 2). This selectivity does not seem to depend on specific sequences.

IFN may block the function of a factor required for formation of a transcription complex. Two interesting candidates, whose expression is decreased by IFN treatment, are histones(159,160) and the c-myc protein products (196-198). On the other hand, the introduced DNA may form transcriptional complexes that could form dsRNA and activate the (2'-5')oligoadenylate synthetase-RNase L pathway leading to the local degradation of RNA (100).

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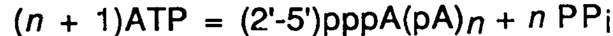
CHAPTER FOUR

STUDIES OF (2'-5')OLIGOADENYLATE BINDING PROTEINS.

INTRODUCTION

(2'-5')oligoadenylate synthetases are latent enzymes, whose level is increased by IFN. Once activated by dsRNA they convert ATP into (2'-5')oligoadenylates (2'-5'A) and pyrophosphate as depicted below (22).



$$(n + 1)\text{ATP} = (2'\text{-}5')\text{pppA(pA)}_n + n\,\text{PP}_i$$

2'-5'A are oligonucleotides of general structure (2'-5')pppA$(pA)_n$, where n varies from 2 to 15 (78). The only known role of 2'-5'A is to bind and activate a 2'-5'A dependent endonuclease, RNase L (22,78,100).

The binding of 2'-5' A to RNase L has been a useful assay to detect this protein in the cytoplasm of mammalian cells. A labeled derivative of 2'-5'A, (2'-5')pppApApApA[32P]pCp, binds and is UV-crosslinked to a 75-80 kD cytoplasmic protein, which co-purifies with the RNase L (119). (2'-5')pppApApApA[32P]pCp is UV-crosslinked to several proteins in nuclear extracts of EAT cells (84,119). The nuclear 2'-5' A binding proteins have unknown activity and function. 2'-5'A binding proteins have been found in

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every mammalian cell and tissue studied, and have been detected in reptilian and amphibian tissues (124). These proteins have not been detected in plant tissues (124), although there is a claim in the literature of (2'-5')oligoadenylate synthetase-like activities in tobacco extracts (124,219).

The 2'-5' A dependent endonuclease activity (RNase L), the 2'-5'A binding activity and a 77 kD protein 2'-5' A-crosslinked product co-purify through several steps. This lends strong support to the idea that these three activities reside in the same polypeptide and, therefore, we will use these terms interchangeably (119,125). Three reports on the partial purification of the cytoplasmic 2'-5'A binding protein, RNase L, have been published (102,118,119,125). Rnase L is present in very low levels in most cells and tissues. The nuclear 2'-5'A binding proteins have not been purified, and their characterization is incomplete (84).

As was discussed in chapter one the (2'-5')oligoadenylate synthetase-RNase L pathway has been implicated in the anti-viral (106,128-132,134-140) and anti-proliferative (141-145) actions of IFNs. Therefore the characterization and manipulation of both the pure protein and the gene for the RNase L are pressing priorities.

In this chapter I will present data on the presence of 2'-5'A binding proteins in different mammalian extracts from cells and tissues. I will describe the characterization and partial purification of a 78,000 dalton 2'-5'A binding activity from calf spleens.

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MATERIALS AND METHODS.

Cells and IFNs. Hamster V79 cells were grown as described in chapter 3. Suspension Hela and Ehrlich Ascites Tumor (EAT) cultures were grown in MEM (spinner) with 10% fetal calf serum (GIBCO) with gentle stirring in spinner flasks at 37°C. IFN preparations were as described in chapter two.

Synthesis of (2'-5')pppApApApA[32P]pCp. This labeled derivative of (2'-5')pppApApApA was synthesized and purified by a modification of the method of Silverman et al. (220). (2'-5')pppApApApA (Pharmacia) was ligated to [32P]pCp(3000 Ci/mmol)(New England Nuclear) with T4 RNA ligase (Pharmacia) at 4°C for 72 h. The ligated product was purified on a high performance liquid chromatography reverse phase C_{18} bondapak column (Waters) using a two pump gradient system (Rainin). A gradient starting with 100% A (50mM Ammonium phosphate (pH 6.9)), 0% B (50mM Amm. phosphate (pH 6.9):methanol, 70:30(v/v)). B increased by 2%/min to a final composition of 30%. The flow rate was 0.5 ml/min. (2'-5')pppApApApA eluted with a retention time of 12.5 min and (2'-5')pppApApApA[32P]pCp with a retention time of 11 min. (2'-5')pppApApApA[32P]pCp was also purchased from Amersham. The products were analyzed using TLC as described below.

Cellular extracts. All procedures in this and all subsequent sections were carried out at 4°C. unless otherwise specified. Method I. Cytoplasmic extracts were prepared as described by Floyd-Smith et al. (119). Essentially washed cells were swollen in hypotonic buffer and disrupted by homogenization in a tight fitting Dounce homogenizer. Cytoplasmic extracts were also

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made as described below in Method II. Method II. Cells in suspension culture were spun at 1600g for 5min and washed twice by resuspending in 10 packed cell volumes of chilled PBS and respining as above. Cells in monolayer culture were washed on the plates with chilled PBS, scraped with a rubber policeman into chilled PBS, spun and washed as above. The washed cells were resuspended in 1ml of PBS and transferred to 1.5 ml Eppendorf tubes, spun at 12,000g for 15 s and resuspended in 2 packed cell volumes of lysis buffer (20mM Hepes-KOH (pH 7.4),10mM KCl, 1. 5mM MgAcetate, and 15mM ß-mercaptoethanol) supplemented with protease inhibitors: 25 to 100 μg/ml leupeptin, 1mM 1,10-phenanthroline, 1 mM benzamidine, 1mM sodium metabisulfite, 100 to 2000 μM PMSF, 50 μg/ml soybean trypsin inhibitor and 50 μg/ml ovomucoid trypsin inhibitor. Nonidet P-40 (NP-40) was added to the cell suspension to a concentration of 0.5% (w/v), and the suspension was incubated on ice for 10 min with vigorous vortexing every min. The cell nuclei were sedimented at 12,000g for 2 min, the supernatant fraction was collected and frozen in aliquots at -80°C.

Nuclear extracts. Nuclear extracts of Hela cells were made as described by Floyd-Smith et al. (119) and modified by St. Laurent et al (84). The nuclear pellet of cells, lysed by hypotonic swelling, was dialyzed against 500mM KCl and the insoluble residue was sedimented at 200,000g. The dialyzed supernatant fraction was used in the assays. Nuclear extracts of Hela cells were also made following the procedure of Dignam (221).

Extracts of calf tissues. (See Table 1 for calf spleen extract). Calf liver, spleen , pancreas, and thymus were obtained,

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immediately post mortem, at Forte's abbatoir (No. Branford, CT) and transported to the laboratory on ice. The organ parenchyma was diced into 1 cm3 pieces, excluding large blood vessels. In the case of the liver and spleen the capsule was dissected away before dicing. The pieces were immersed in 2ml of extraction buffer (20mM Tris-HCl (pH 7.4), 10mM KCl, 1.5mM MgAcetate, 15mM ß-mercaptoethanol, 12% (v/v) glycerol supplemented with 25 to 100 µg/ml leupeptin, 1mM 1,10-phenanthroline, 1 mM benzamidine, 1mM sodium metabisulfite, 100 to 2000 µM PMSF, 50 µg/ml soybean trypsin inhibitor and 50 µg/ml ovomucoid trypsin inhibitor to reduce proteolysis) per gram of tissue (223). The tissue was homogenized in a Virtis double blade homogenizer at 2000 to 3000 rpm for three to five 20-sec bursts. The thick homogenate was spun in a Sorvall GSA rotor at 3000 rpm for 10 min and the supernatant fraction spun again in the same rotor at 10,000 rpm for 30 min. The second low speed supernatant fraction (S10) was spun at 170,000g for 3 h and the 170,000g supernatant fraction (S200) was aliquoted and frozen at -80°C.

The (2'-5')pppApApApA[32P]pCp binding assay. The binding assays were carried out as described by Slattery et al (114) and modified by Knight et al (115). The final volume of the assays was 50 to 100 µl. 330 pM (2'-5')pppApApApA[32P]pCp (3000 Ci/mmol)was incubated with extracts in 25mM Tris-HCl (pH 7.4), 10mM MgAcetate and 30mM ß-mercaptoethanol. The assays were incubated at 4°C for 3 h ,unless otherwise indicated, and the binding solution was filtered through nitrocellulose filters (Millipore, pore size = 45µ) which had been presoaked for 5 min in wash buffer

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(25mM potassium phosphate (pH 7.4), 50mM KCl, 5mM MgAcetate, 30mM ß-mercaptoethanol) supplemented with 4% (w/v) sodium pyrophosphate. The filters were washed with 5 ml of wash buffer, air dried and counted in a scintillation counter (Searle), with or without scintillation fluid.

(2'-5')pppApApApA[32P]pCp crosslinking assay. The (2'-5')pppApApApA[32P]pCp and the extracts were incubated as described for the binding assays, the binding solutions were transferred to separate wells in a 96-well tissue culture dish (Corning) and irradiated with a germicidal UV lamp at 254 nm for 1 h for a total radiation dose of 36,000 J/m2 as described by Floyd-Smith et al. (119) Crosslinked products were fractionated on SDS-polyacrylamide gels and visualized by autoradiography as described in chapter two. Typical exposure times were between 24 and 48 h at -70°C.

2'-5'A dependent endonuclease assays. The S200 of calf spleens was diluted in extraction buffer and incubated with uniformly labeled SV40 early RNA (transcribed in vitro using the SP6 promoter/polymerase system in the presence of [32P]UTP and kindly donated by M. Payne and Dr. P.K. Ghosh) in 20mM Tris-HCl (pH 7.6), 80mM KCl, 4mM MgAcetate and 5% (v/v) glycerol. 84 µM (2'-5')pppApApApA was added and the assays were incubated for 30 min at 30°C. RNA products were visualized by electrophoretic fractionation on denaturing acrylamide gels (213) followed by autoradiography.

Thin layer chromatography. Analytical ascending thin layer chromatography on polyethyleneimine (PEI)-cellulose plates

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followed the procedure of Samanta et al (81); 1 μl samples were loaded and air dried; plates were then developed with either 0.75 M KH_2PO_4-HPO_4 (pH 3.7) or with freshly made 0.125M ammonium bicarbonate. Plates were air dried and separated products were visualized by autoradiography.

Pre-affinity Purification Schemes. (See tables 2 and 3) The details of individual purification steps will be given in the results section, here I will introduce information about general methods and materials used.

Protein concentrations was determined with the Bio-Rad assay following the specifications of the manufacturer. Standard curves were constructed using bovine serum albumin (Miles).

Conductivity of samples was measured using a Radiometer Copenhagen conductivity meter, and using a KCl standard curve. pH was measured with a Corning pH meter, except with solutions containing high protein concentration for which pH paper was used.

Ammonium sulfate cuts. Ammonium sulfate and ammonium bicarbonate were of the best quality available, and were used fresh. The amount of ammonium sulfate:ammonium bicarbonate (50:1 w/w) mixture to be added to the S200 was calculated from the formula grams/ L = $[533(S_2-S_1)]/[100-0.3S_1]$ where S_1 and S_2 are the initial and final concentrations of ammonium sulfate in grams per 100 ml (224).

DEAE-anion exchange chromatography. DE-52 (Whatman) and DEAE-Sephacel (Pharmacia) were used interchangeably without any significant effect on the purification . The matrices were equilibrated following the recommendations of the manufacturers,

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poured as a slurry in buffer A (20mM Tris-HCl (pH 7.5), 10mM ß-mercaptoethanol, 40µM EDTA, 10% (v/v) glycerol) and packed at a flow rate of 11-12 ml/hr-cm2. After use the columns were washed with buffer A supplemented with 2M KCl, repoured and repacked before each use.

Phosphocellulose matrix. P-11 (Whatman) was regenerated following the recommendations of the manufacturer. The matrix was equilibrated to pH 7.5 with 100mM Tris-HCl (pH 7.5), resuspended in a slurry with buffer A and poured into a column. P-11 was packed at a low flow rate. New matrix was used for each separation.

Phenyl sepharose-hydrophobic interaction chromatography (225). Phenyl sepharose (Pharmacia) was washed in double distilled, autoclaved water to eliminate the ethanol in which the matrix is shipped. The matrix was packed in buffer A supplemented with 1M KCl at a flow rate of 10 ml/hr-cm2. New matrix was used for each separation.

Affigel Blue - Blue dye chromatography (226). Affigel blue matrix (Bio-Rad) was poured as a slurry in buffer A, poured and packed at flow rates of 12 ml/hr-cm2. After use the matrix was regenerated by washing in 2M KCl followed by washing in water.

RESULTS

2'-5'A binding proteins in nuclear and cytoplasmic extracts of mammalian cells in culture. We prepared cytoplasmic extracts (method I in Materials and Methods) from EAT cells treated with 500 U/ml of mouse ß IFN for 18 h. We detected

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in these extracts a 2'-5'A binding activity using the binding assay of Slattery (114) as modified by Knight (115). The binding of proteins to radioactively labeled (2'-5')pppApApApA[32P]pCp, a derivative of 2'-5'A, is detected by the selective retention of bound counts on nitrocellulose filters. Binding increased linearly with increasing amounts of extract added to the binding assays, (not shown), and the binding dependence on [(2'-5')pppApApApA[32P]pCp] permitted us to calculate a $K_a = 1 \times 10^{-10}$ M (not shown).

UV-crosslinking of 2'-5' A to protein followed by electrophoretic separation on SDS-polyacrylamide gels and autoradiography revealed only one band with an apparent molecular weight of 78 kD in agreement with the report by Floyd-Smith et al (119), (Fig. 11, lane 1).

When cytoplasmic extracts from other cell lines, human JK cells, a lymphoblastoid line, (Fig 11, lane 2), Hela cells (lane 3) and Chinese hamster V79 cells (lane 4), were crosslinked to (2'-5')pppApApApA[32P]pCp only one crosslinked product (**L** in Fig. 11) was detected with a molecular weight near 78 kD.

UV-crosslinking of (2'-5')pppApApApA[32P]pCp to Hela cell nuclear extracts followed by gel-electrophoresis revealed a more complex picture. A 0.5M KCl salt wash of Hela cell nuclei, isolated following the procedure described by St. Laurent et al (84), contained several (2'-5')pppApApApA[32P]pCp crosslinked proteins (Fig. 11, lane 5:**b-e**) with approximate m.w. of 41-50 kD (**b**),67 kD (**c**),79 and 82 kD doublet (**d**) and 101 kD (**e**). A nuclear extract made following the procedure of Dingam et al (221) contained four crosslinked proteins (Fig 11, lanes 6 & 7: **a,b,d,e**)with m.w. of 22

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Figure 11. 2'-5'A binding proteins in cytoplasmic and nuclear extracts of mammalian cells in culture. (2'-5')pppApApApA[^{32}P]pCp was crosslinked using UV light (see text) to proteins in cytoplasmic extracts of IFN treated EAT cells (lane 1), human JK cells (lane 2), Hela cells (lane 3) and chinese hamster V79 cells (lane 4); **L** identifies the major crosslinked product migrating as a 78 kD protein. (2'-5')pppApApApA[^{32}P]pCp was crosslinked using UV light to proteins in a nuclear extract, from human alpha IFN treated Hela cells , made as described by St. Laurent et al. (84) (lane 5), or to proteins in nuclear extracts made as described by Dingam et al (221) from Hela cells (lane 6) or IFN treated Hela cells (lane 7). Major crosslinked proteins are identified as **a-e** (see text). Protein size markers (for lanes 5-7) are **A**, lysozyme (14.3 kD); **B**, carbonic anhydrase (30 kD); **C**, ovalbumin (46kD); **D**, bovine serum albumin (69 kD); **E**, phosphorylase b (92.5 kD).

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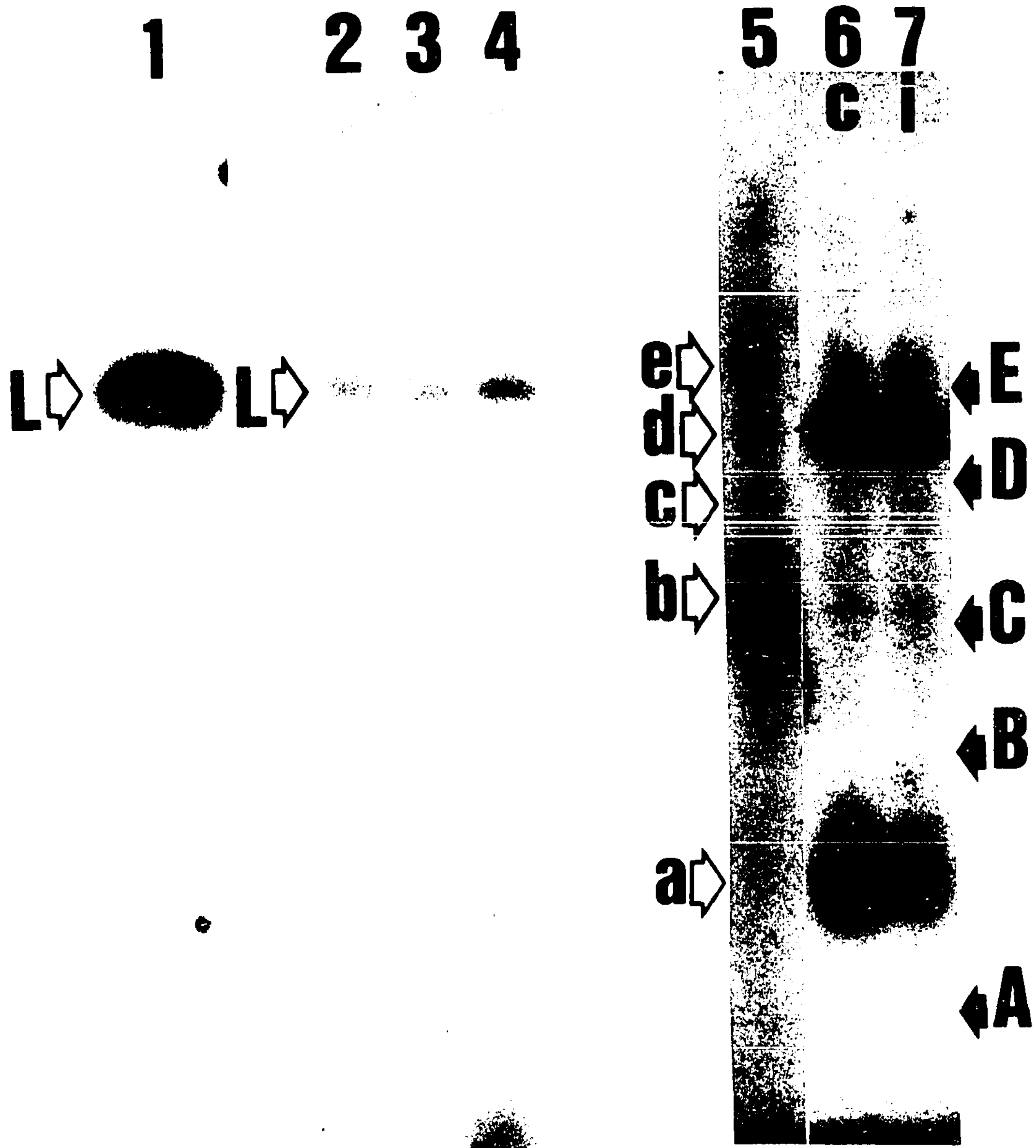

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kD (Fig 11, lanes 6 & 7: (**a**), 50 kD (**b**), 79 kD (**d**), and 101 kD (**e**) . Some of these molecular weights are similar to those reported by St Laurent et al (84) in an EAT nuclear salt wash. As with the EAT proteins, the function(s) of these proteins remains unknown. The 22 kD 2'-5' A binding protein was present in lower amounts in nuclear extracts made from IFN treated Hela cells (lane 7).

Purification of a cytoplasmic 2'-5'A binding protein: General considerations. We decided to undertake the purification of the cytoplasmic protein that binds 2'-5'A, which has been assumed to be the 2'-5'A dependent endonuclease, RNase L. The strategy employed was to divide the purification into three problems to be solved: 1) First, was the selection of a starting material. The selection of a starting material was based on practical considerations: low cost, reliable availability, ease of handling and high yield of the activity. These requirements were fullfilled better by organs than cells in culture. 2) Pre-affinity purification to purify away other nucleases that degrade 2'-5'A, and make a 2'-5'A affinity column useless. 3) The synthesis and use of an affinity column based on the high affinity of the protein to 2'-5'A. We felt that the first and third problems would be the most challenging ones; and would hold the key to the purification. The best purification to date was reported by Floyd-Smith and Lengyel, and we owe to this work many ideas (119,125).

The assays used during the purification were the 2'-5'A binding and crosslinking assays because our goal was the purification of a 2'-5'A binding protein . We assumed that there was a very good chance that the 2'-5'A dependent endonuclease

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activity resided in the same protein (118,119,125).

Purification of a cytoplasmic 2'-5'A binding protein: The selection of the starting material. Two sources of organs were tested: first, human placenta, the only human organ readily available, and second, bovine organs. We made low speed supernatants (Table 1) of human placenta, and calf spleen, thymus, liver, and pancreas . For comparison we made a similar extract from IFN treated EAT cells. Extracts were made as described in the materials and methods (Table 1), tissues were dissected minced and homogenized in extraction buffer supplemented with protease inhibitors using a Virtis double blade homogenizer. The supernatant of the second low speed spin (S10 in Table 1) was used in the assays.

The spleen extract was the richest in 2'-5'A binding activity, with about 4-fold higher binding activity per µg of total protein than the extract from IFN treated EAT cells (Fig. 12A). Assuming a 1:1 binding of the protein and the 2'-5'A, we calculated that there are between 5 and 10µg of this 2'-5'A binding protein per gram of total protein in the S10 of calf spleens. Therefore this protein would have to be purified 100,000 to 200,000 fold in order to obtain homogenous RNase L.

Human placenta was the only tissue which did not have detectable binding activity (Fig. 12A); however, this could have been due to a very strong (2'-5')pppApApApA[32P]pCp degradation activity we found in this tissue (not shown).

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Table 1

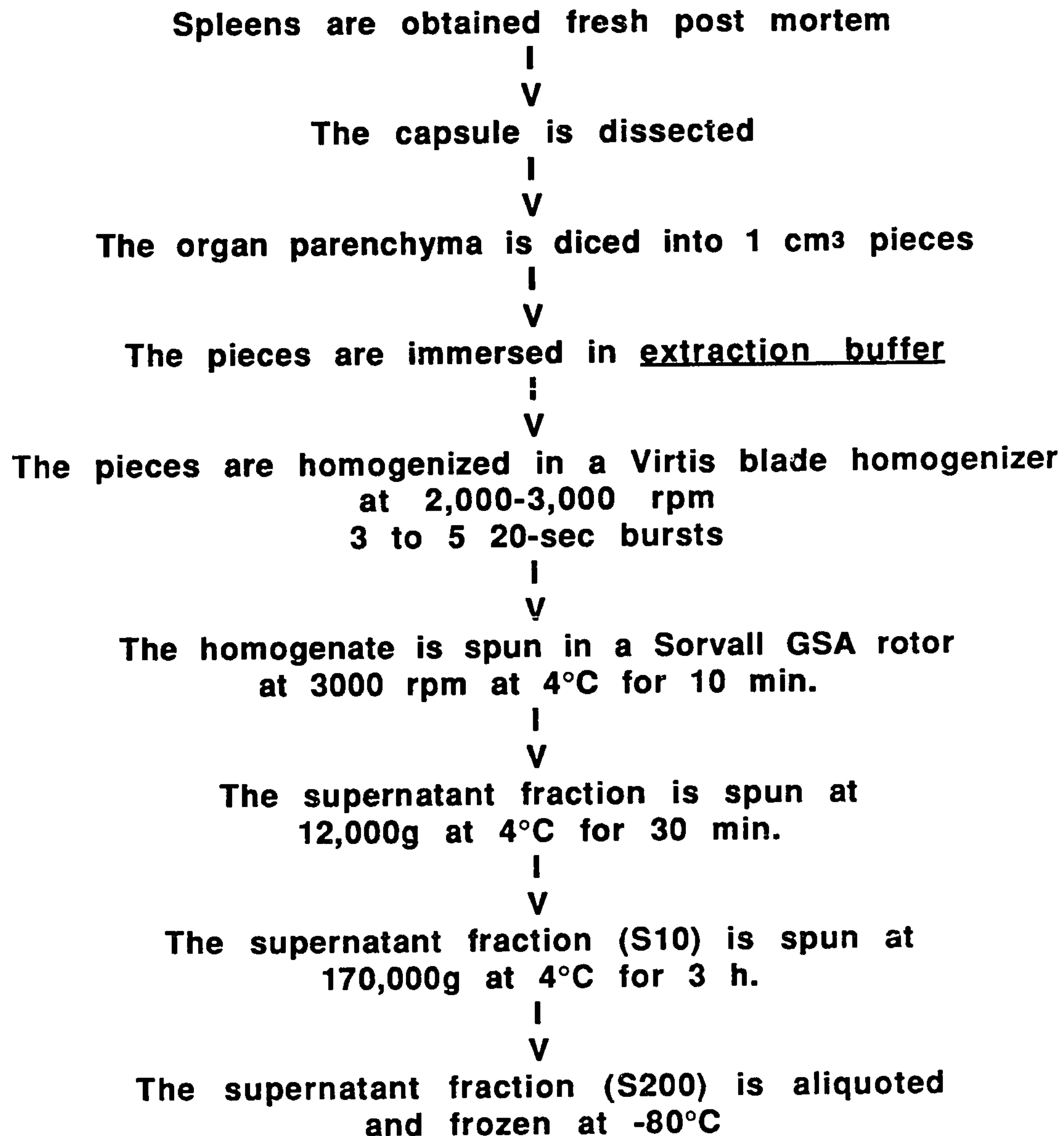

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Figure 12. 2'-5'A binding proteins from cytoplasmic extracts of calf and human tissues. A. Binding assays were carried out with 50μg of total protein of tissue extracts (see text). The amount of bound (2'-5')pppApApApA[32P]pCp is shown. B. Crosslinking of (2'-5')pppApApApA[32P]pCp to proteins in a preparation of bovine serum albumin (lane 1), cytoplasmic extract from IFN treated EAT cells (lane 2), calf spleen extract (S10 in Table 1) (lane 3), calf thymus extract (lane 4), calf liver extract (lane 5), calf pancreas extract (lane 6), and human placental extract (lane 7). The major crosslinked product (**L**) for all the extracts co-migrated. Spleen extracts had at least two other minor crosslinked products (**a**). Protein size markers are carbonic anhydrase (30 kD), **A**; ovalbumin (46 kD), **B**; bovine serum albumin (69 kD), **C**; phosphorylase b (92.5 kD), **D**; and myosin (200 kD), **E**.

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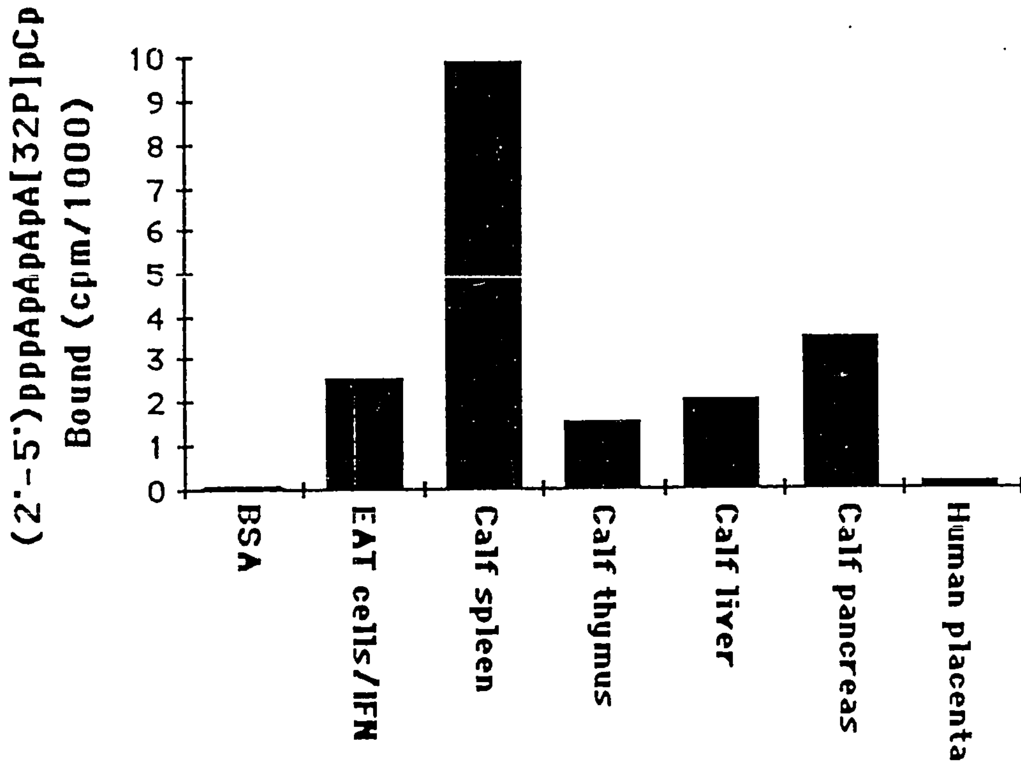
A



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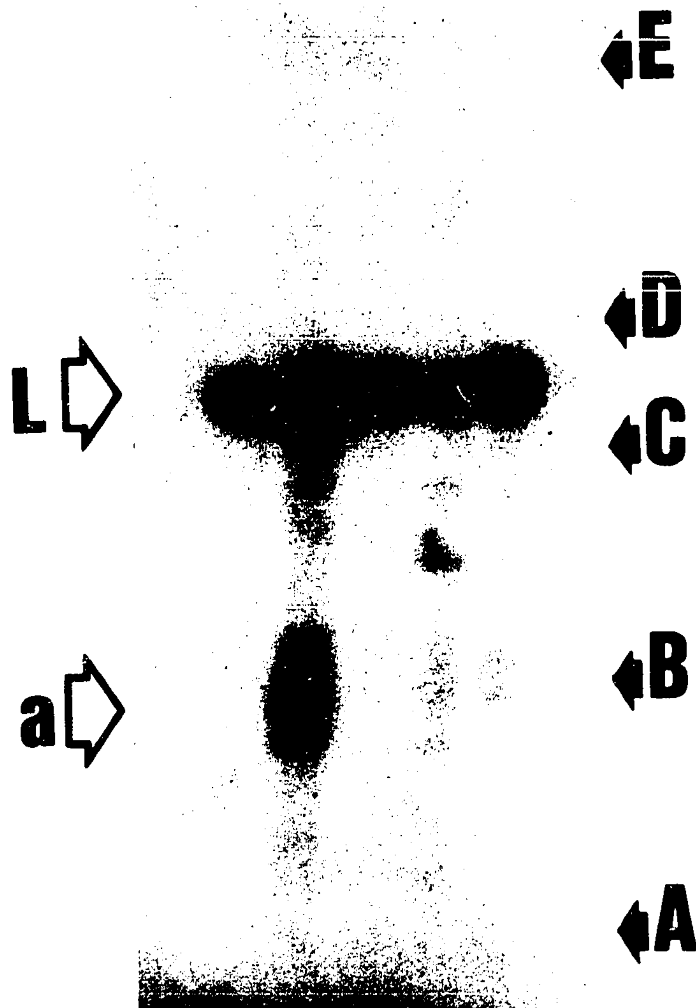
B

1 2 3 4 5 6 7



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All the extracts, except human placental extract, contained a protein that was UV-crosslinked to (2'-5')pppApApApA[32P]pCp (Fig. 12B, lanes 2-7). Spleen extracts (lane 3) had the highest level of (2'-5')pppApApApA[32P]pCp crosslinked material, which migrated as a broad band with an apparent m.w. of 75-79 kD (**L** in Fig. 12B) in SDS-polyacrylamide gels. This is compared with EAT (lane 2), calf thymus (lane 4), calf liver (lane 5), calf pancreas (lane 6) and human placenta (lane 7). A minor crosslinked product in calf spleen migrated with an apparent m.w. of 40-45 kD (lane 3:**a**). The ratios of binding to crosslinking were not uniform, as has been noted by others (119). The major crosslinked proteins in all the tissues are of similar molecular weight and all the tissues seem to contain a broad band of crosslinked material (Fig. 12B). The EAT cytoplasmic extracts contain only one crosslinked protein (Fig. 12B, lane 2) as was previosly reported (119).

We decided to use calf spleen extracts as starting material for the purification of the 2'-5'A binding protein.

Purification of a 2'-5'A binding protein: Pre-affinity purification. 1) Characterization of the 2'-5'A binding activity in calf spleens. The second low speed supernatant (S10) and pellet fractions and the high speed supernatant (S200) and pellet fractions were analyzed for 2'-5'A binding and crosslinking (Fig. 13A, lanes 1 & 3). The S200 (lane 3) had 3 fold higher levels of the 75-79 kD 2'-5'A binding protein per gram of total protein than the S10 (lane 1); but given that the total protein concentration was diminished only by 1.86 fold this implies

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Figure 13. Purification of a calf spleen 2'-5'A binding protein: differential centrifugation. **A.** (2'-5')pppApApApA[32P]pCp was crosslinked to 50 µg of protein in the supernatant fraction (S10) (lane 1) or the pellet fraction (lane 2) obtained from the second low speed spin (Table 1), or to proteins in the supernatant fraction (S200) (lane 3) or pellet fraction (lane 4) of the high speed spin (Table 1). The 75-79 kD crosslinked protein is labeled **L**, a 44 kD protein that preferrentially sedimented in the high speed spin is identified with arrows (lanes3 & 4). Protein size markers are ovalbumin (46 kD), bovine serum albumin (69 kD), and phosphorylase b (92.5 kD). **B.** The stability of (2'-5')pppApApApA[32P]pCp was determined by incubating it with the extracts under binding assay conditions and visualization by autoradiography of [32P] containing compounds fractionated by TLC (see text). (2'-5')pppApApApA[32P]pCp incubated in the absence of extract (lane 1), incubated with the supernatant (S10) (lane 2) or pellet (lane 3) fractions of the low speed spin, or incubated with the supernatant (S200) (lane 4) or pellet (lane 5) fractions of the high speed spin. (2'-5')pppApApApA[32P]pCp and (2'-5')ppApApApA[32P]pCp, and two degradation products (**a** and **b**) are identified. Origin (**o**); solvent front (**f**).

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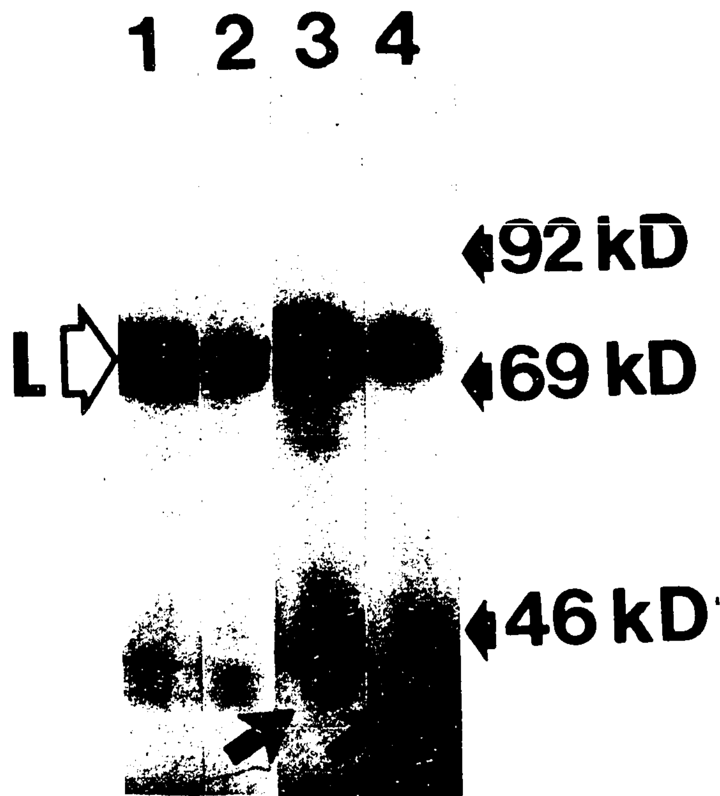

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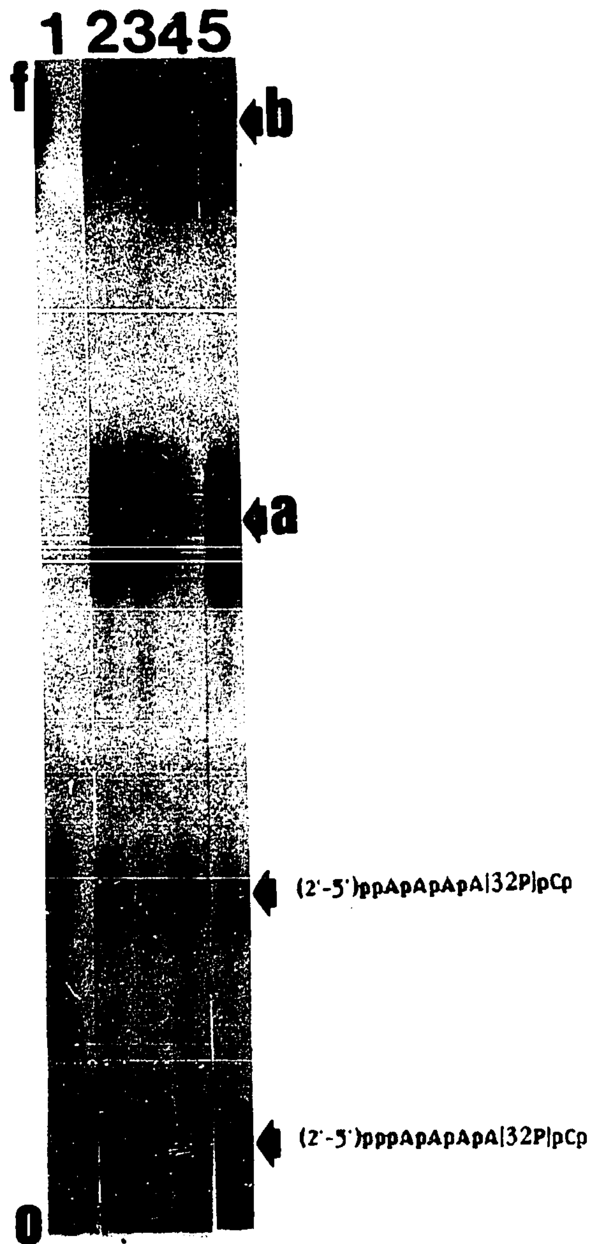

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a recovery of over 100% yield. This may be explained by degradation of 2'-5'A in the S10.

Most of the (2'-5')pppApApApA[32P]pCp added to the S10 was degraded (Fig. 13B, lane 2). In the S200 (2'-5')pppApApApA[32P]pCp was more stable (lane 4), with most of the degradative activity sedimenting with high speed pellet (lane 5).

One extraction was carried out with all protease inhibitors mentioned above except leupeptin. Although we obtained a high yield of binding activity per mg of total protein, the crosslinked material had low molecular weights (not shown). A similar dependence on leupeptin for stability of the 2'-5'A binding proteins was seen by Krause et al (143,144).

Interestingly, a small crosslinked product of m.w. 44 kD was preferrentially sedimented during the high speed spin (Fig. 13A, lanes 3 & 4, solid arrows). The binding activity in the resuspended pellet was divided equally between the 75-79 kD band and this 44 kD band (lane 4).

The resuspended pellet fraction bound (2'-5') pppApApApA[32P]pCp with a linear dose dependence for the extract (not shown); the binding saturated at subnanomolar concentrations of (2'-5')pppApApApA[32P]pCp, and had a $K_a = 1.5 \times 10^{-11}$ M (not shown). Furthermore, the binding was completely inhibited by the addition of 10nM (2'-5')pppApApApA, but was not affected by 2mM ATP (not shown).

We decided to focus our studies on the 75-79 kD protein in the S200. The bulk of the 2'-5' A binding activity in the S10 remained in the S200. The 2'-5'A binding in the S200 increased

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linearly with dose of extract (Fig. 14A). The binding had a K_a of between 10^{-10} and 10^{-11}M (Fig 14B). The binding to (2'-5') pppApApApA[32P]pCp was inhibited completely by addition of 1nM 2'-5'A, with a $K_i = 1 \times 10^{-11}$ M (Fig. 14C); while other nucleotides (eg; ATP and pCp) even at mM concentrations did not compete with the binding.

The binding reaction was very fast at 4°C, with over 75% of maximal binding occurring by 1 min, and once bound the complex was stable for over 72 h (not shown). The exchange reaction was determined to be extremely slow, with less than one third of the bound 2'-5'A exchanged in 60 h (not shown).

The S200 contained a 2'-5'A dependent ribonuclease activity. Uniformly labeled SV40 RNA was digested more efficiently by S200 in the presence of (2'-5')pppApApApA (Fig. 14D, lane 4), than in its absence (lane 3). The activity was measured by observing the disapearance of the labeled transcript in 8M Urea-acrylamide gels (Fig. 14D). Dilutions of the S200 had to be used in order to detect 2'-5' A dependent nuclease activity over other nuclease activity.

2) Purification scheme I (Table 2). Ammonium sulfate precipitation. 200 ml of S200 containing 4 grams of protein were precipitated by slow addition of an ammonium sulfate: ammonium bicarbonate (50:1 w/w) mixture with stirring. Once the salt had dissolved the solution was stirred for 30 min and spun at 9000 rpm in SS34 rotor (Sorvall) for 30 min. The ammonium sulfate cuts were at 0-15%, 15-30%, 30-45%, 45-55%, 55-65%, and 65-75% ammonium sulfate. The pellets from the first three cuts, 0-45%,

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Figure 14. Characterization of the 2'-5'A binding protein in the supernatant fraction of the high speed spin of calf spleens. **A.** Binding assays were carried out with increasing amounts of supernatant fraction from the high speed spin (Table 1). The bound (2'-5') pppApApApA[^{32}P]pCp is plotted vs. concentration of the supernatant (extract). **B.** Binding assays were carried out using10μl of supernatant fraction and varying amounts of (2'-5')pppApApApA[^{32}P]pCp. The bound (2'-5')pppApApApA[^{32}P]pCp is plotted vs. [(2'-5')pppApApApA[^{32}P]pCp]. **C.** Inhibition of (2'-5')pppApApApA[^{32}P]pCp binding by addition of (2'-5')pppApApApA to binding assays. The bound (2'-5')pppApApApA[^{32}P]pCp is plotted vs. [(2'-5')pppApApApA]. **D.** Uniformly labeled SV40 early RNA was incubated with dilutions of the high speed supernatant (S200) of calf spleen extracts in the presence of 80μM (2'-5')pppApApApA for 15 min at 30°C. The labeled RNA was fractionated in sequencing gels and visualized by autoradiography. This is shown for RNA incubated with a 1:10^3 dilution of S200 without (lane 1) and with (2'-5')pppApApApA (lane 2); with a 1:10^4 dilution of S200 without (lane 3) and with (2'-5')pppApApApA (lane 4); with a 1:10^5 dilution of S200 without (lane 5) and with (2'-5')pppApApApA (lane 6); or RNA incubated with (2'-5')pppApApApA without S200 (lane 7).

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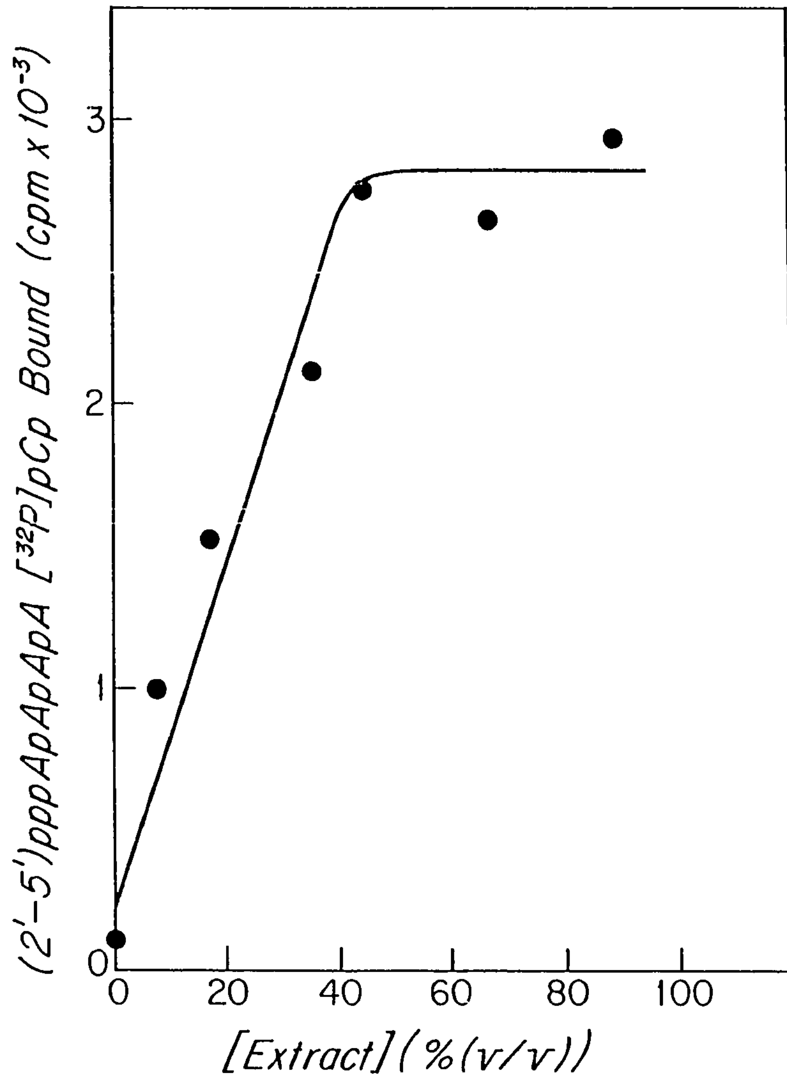

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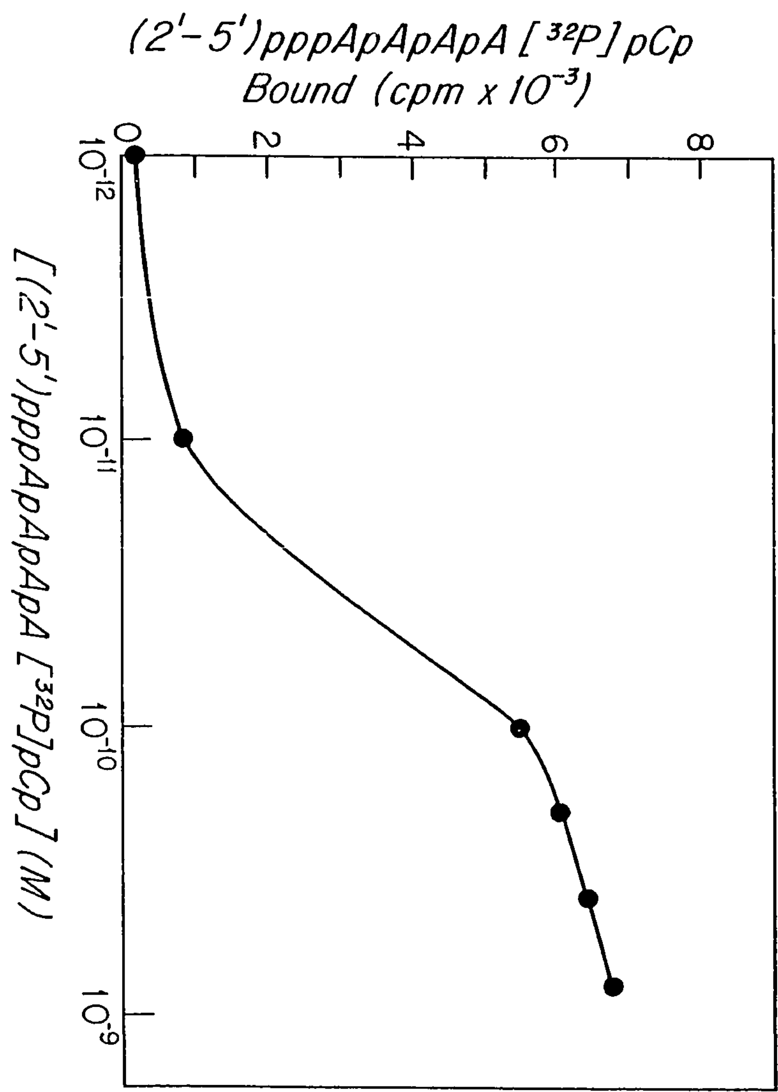

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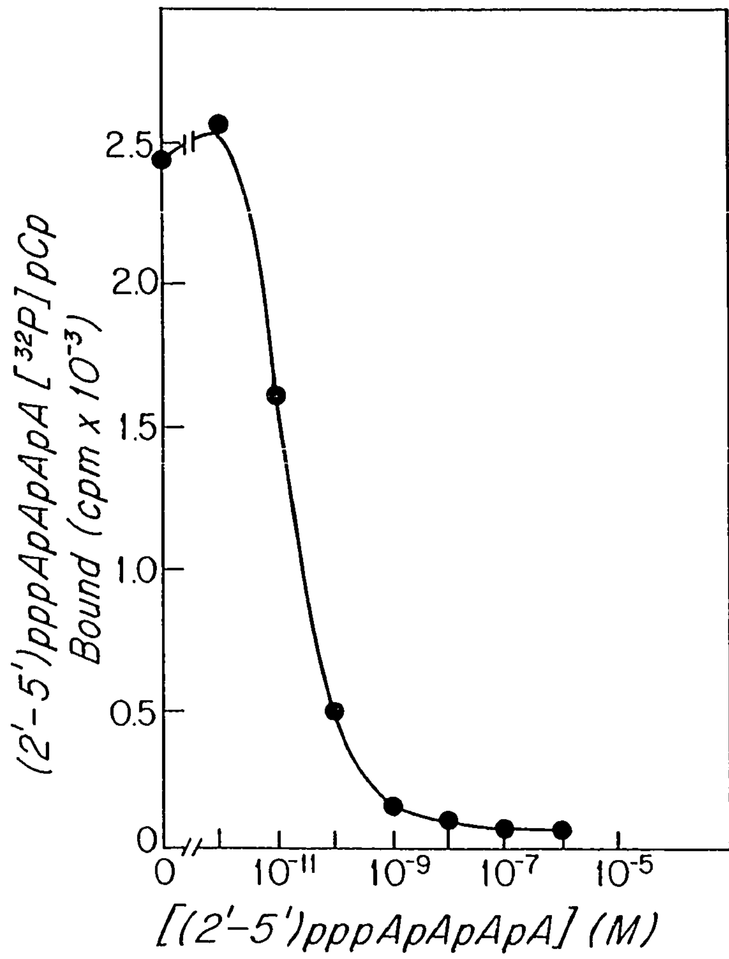

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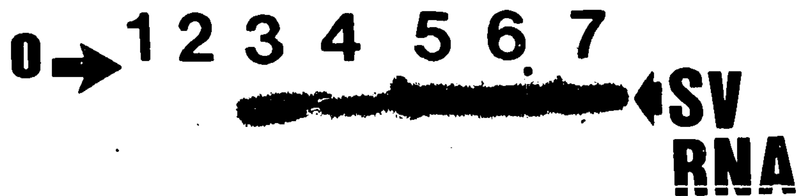

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Table 2. Purification of a cytoplasmic 2'-5'A binding protein from calf spleens. Purification scheme I.

Step	Total protein	Yield[1]	Purification fold[2]
S200	4,000.0 mg	100.0%	1.0
AS	1,000.0 mg	30.0%	1.2
DE-52	497.0 mg	27.0%	2.5
P-11	7.7 mg	22.0%	100.0
Phenyl sepharose[3]	0.65 mg	5.3%	286.0

1. The yield was calculated from the total number of moles of (2'-5') pppApApApA[32P]pCp bound. This was calculated from the specific activity of the (2'-5')pppApApApA[32P]pCp and the number of cpm bound. The numbers given are for overall yield.
2. Purification fold was calculated from the ratio of (2'-5') pppApApApA[32P]pCp binding protein to total protein (w/w). The molecular weight of the (2'-5')pppApApApA[32P]pCp binding protein was assumed to be 80,000 daltons.
3. Only 3.5 mg of the P-11 purified material was loaded on this column.

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(AS I) and the last three cuts,45-75%, (AS II) were resuspended in buffer A, dialyzed against this buffer, assayed and respectively pooled together. The AS II had most of the recovered crosslinked protein (Fig. 15, S200, lane 1; AS I, lane 2: AS II, lane 3), this was purified 1.2 fold with a 30% yield (Table 2).

DEAE anion exchange chromatography. 200 ml of the AS II fraction was dialysed, and diluted 1:1 with buffer A supplemented with protease inhibitors This was loaded on a 220 ml DE-52 column at a flow rate of 4.7 ml/h-cm2. The loaded material contained 1 g of protein, at a concentration of 5 mg/ml, a pH of 7.5 and a conductivity equivalent to 37.5mM KCl. The column was washed with 800 ml of buffer A supplemented with 50mM KCl, and was developed, in buffer A supplemented with protease inhibitors, with a 800 ml linear gradient from 50mM to 500mM KCl; both at 4.7 ml/h-cm2. The binding activity eluted in two overlapping peaks, DEAE-I and DEAE-II (not shown); which contained indistinguishable crosslinked proteins (not shown). The binding activity in DEAE-I was 2-fold purified over the AS-II, with a recovery of 69% (Fig. 15)(Table 2).

Phosphocellulose matrix chromatography. The DEAE-I fraction was diluted 1:1 with buffer A with protease inhibitors, to a final volume of 226 ml containing 497 mg of protein, and loaded on a 150 ml phophocellulose (P11) column at a flow rate of 4.1 ml/h-cm2. The loaded material had a protein concentration of 2.2 mg/ml, pH of 7.5 and a conductivity equivalent to 75mM KCl. The column was washed with 450 ml of buffer A supplemented with 75mM KCl and developed with a linear gradient from 75mM to 1M KCl

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Figure 15. Purification of a 2'-5'A binding protein from calf spleens. (2'-5')pppApApApA[^{32}P]pCp was crosslinked to proteins in S200 and in several purified fractions. The crosslinked products were fractionated using SDS-PAGE and visualized by autoradiography. This is shown for the S200 (lanes1 & 4), ASI (lane 2), ASII (lane 3 & 5), see text, DEAE I (lane 6) see text, the peak fraction of P-11, scheme I (lane 7) see text, and the peak fraction of phenyl sepharose column (lane 8), see text. The major crosslinked product migrated as a protein of 78 kD (**L**).

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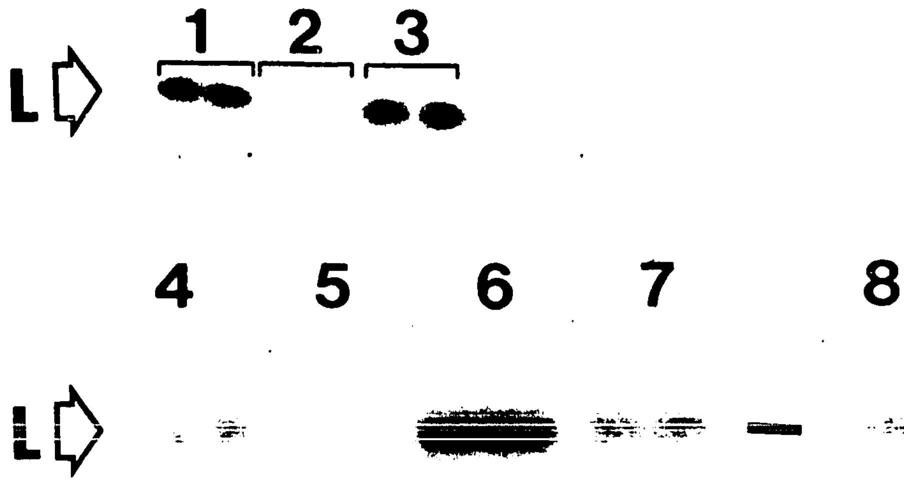

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at a flow rate of 4.1 ml/h-cm2. The binding activity eluted as a peak, with a trailing edge (not shown). Several fractions were pooled, P11-I. This contained 85% of the loaded binding activity, but only 7.5 mg of protein; giving a 45-fold purification of the 2'-5'A binding protein over the DEAE-I fraction (Fig. 15, lane 7, and Table 2).

Phenyl-sepharose, hydrophobic interaction chromatography. 47 ml of the P11-1 fraction was supplemented with 1M KCl and loaded on a 0.9 ml phenyl sepharose column at a flow rate of 2.5 ml/h-cm2. The loaded material contained 3.5 mg of protein; and was only one half of the P11-I fraction. The column was developed with step washes of buffer A with protease inhibitors supplemented with: a) 0.5M KCl , b) 0.1M KCl , c) no salt , d) 1% (w/v) NP-40 (not shown). The binding activity eluted as a sharp peak with the 1% NP-40 wash (Fig. 15, lane 8).

The overall purification of this scheme was approximately 286 fold with a yield of 5.3%. The 2'-5'A binding protein maintained a stable molecular weight throughout the purification (Fig. 15).

The active fractions that eluted from phenyl sepharose had a reduced (2'-5')pppApApApA[32P]pCp degradative activity; but most of the (2'-5')pppApApApA[32P]pCp was still partially dephosphorylated (not shown).

3) Purification scheme II (Table 3). A second scheme of purification was developed with the principal aim of obtaining a partially purified fraction which would not degrade (2'-5')pppApApApA[32P]pCp.

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Table 3. Purification of a cytoplasmic 2'-5'A binding protein from calf spleens. Purification scheme II.

Step	Total protein	Yield[4]	Purification fold[1]
S200	4,000.0 mg	100.0%	1.0
Affigel Blue	101.5 mg	29.9%	10.9
P-11	7.8 mg	14.8%	70.6

[4] See Table 2.

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Affigel blue matrix. 200 ml of S200 supplemented with 250mM KCl, were loaded on a 110 ml affigel blue column, at a flow rate of 4.42 ml/h-cm2. The loaded material contained 4.6 g of protein, at a concentration of 23 mg/ml and pH of 7.5. The column was washed with 350 ml of buffer A supplemented with 250 mM KCl, and developed in the same buffer with a linear gradient from 250mM to 2.5 M KCl. The gradient fractions were assayed and pooled into four pools (E1-E4) and two pools (E3 & E4) were mixed and used for further purification. These two pools contained 29.9% of the loaded binding activity and this was purified 10.9 fold (Fig. 16A and Table 3).

Phosphocellulose matrix. 175 ml of pooled affigel blue purified material (E3+E4), containing 101.5 mg of protein, were dialyzed and loaded on a P-11 column as described above. The column was washed and developed as described above, the binding activity eluted in one peak (Fig. 16B). The peak fractions contained a 6.5 fold purified binding activity with a recovery of 49.5% (Table 3).

The overall purification of this scheme was 70.6 fold with a yield of 14.8%.

Degradation of (2'-5')pppApApApA[32P]pCp. We developed conditions under which (2'-5')pppApApApA[32P]pCp was stable when incubated with fractions purified through scheme II. Incubation of (2'-5')pppApApApA[32P]pCp with P-11 purified fractions in the presence of 200mM potassium phosphate (Fig. 16C, lane 5) or 5mM ATP (lane 7), inhibited the degradation of (2'-5') pppApApApA[32P]pCp (lanes 1 & 6) to very low levels. The P-11

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Figure 16. Purification of a 2'-5'A binding protein from calf spleens: Pre-affinity purification scheme II. <u>A.</u> Fractions eluting from Affigel Blue (BioRad) were assayed for activity in the 2'-5'A binding assays. Fractions were pooled according to the ratio of binding activity to total protein. Flow through and wash , **FT/W**; 1st eluate, **E1**; 2nd eluate, **E2**; 3rd eluate, **E3**; and 4th eluate, **E4** (see text). Bound (2'-5') pppApApApA[^{32}P]pCp (black columns) is plotted for each pooled fraction . Total protein (gray columns) is also shown (see text). **<u>B.</u>** Fractions eluted from P-11 were assayed for 2'-5'A binding. Bound (2'-5') pppApApApA[^{32}P]pCp is plotted against fraction number. **<u>C.</u>** (2'-5') pppApApApA[^{32}P]pCp was incubated with P-11 purified fractions under conditions for binding. 1μl of the binding reactions was spotted on PEI-cellulose TLC plates that were developed with KH_2PO_4 buffer (pH 3.7) as described in the text. Binding reactions without added potassium phosphate (lanes 1 & 6), supplemented with potassium phosphate to 25 mM (lane 2), 50 mM (lane 3), 100 mM (lane4), 200 mM (lane 5) or with 5 mM ATP (lane 7). Degradation product (**a**) of (2'-5') pppApApApA[^{32}P]pCp, origin, (**o**); solvent front, (**f**).

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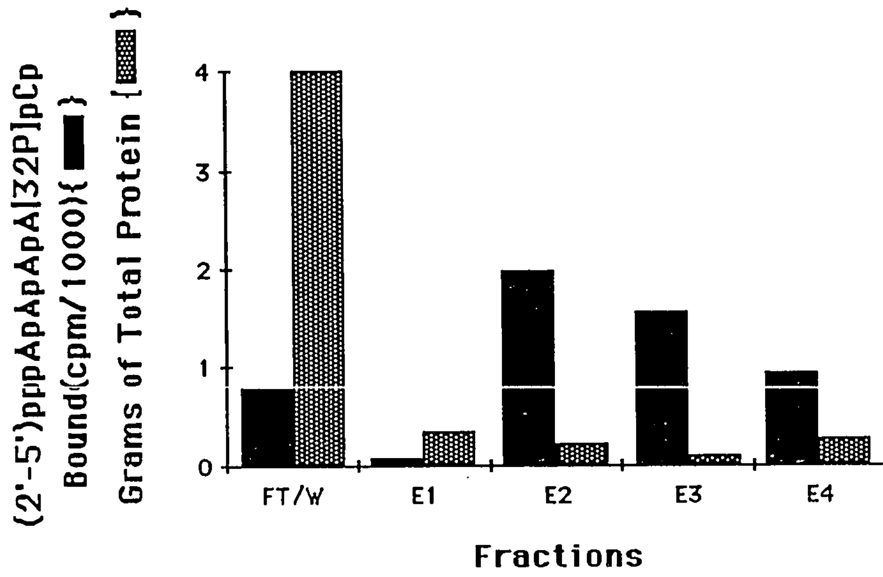

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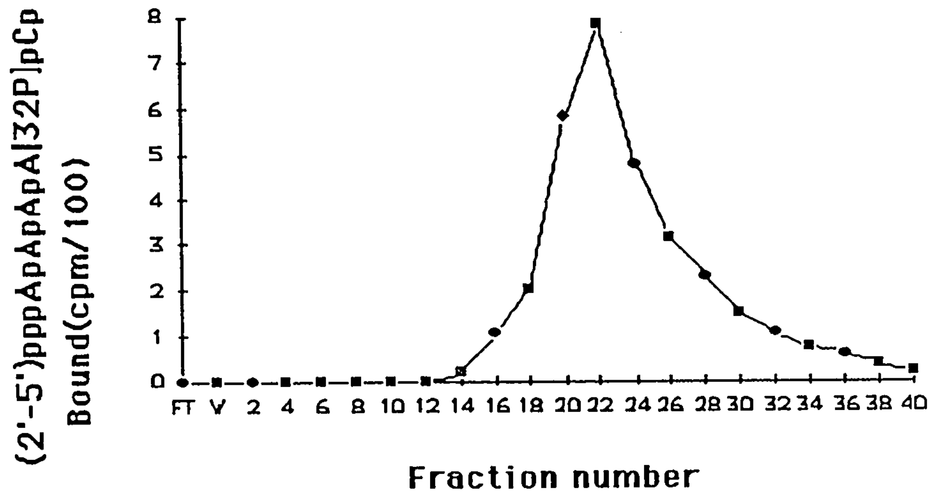

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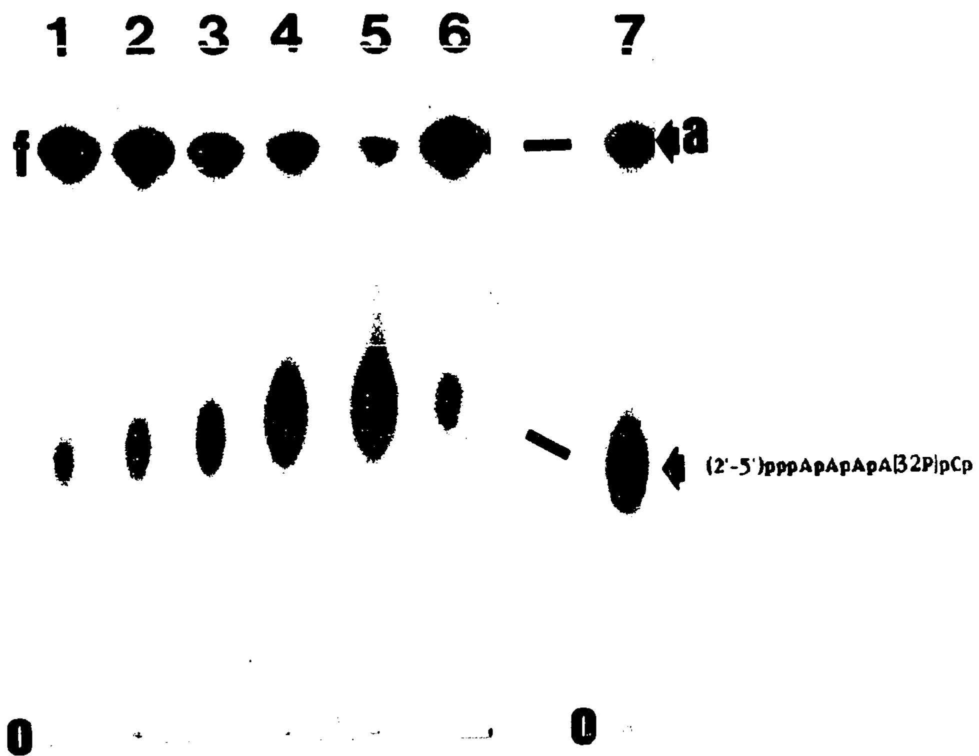

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purified fractions were divided into two pools, I and II, acording to whether these would degrade (2'-5')pppApApApA[32P]pCp in the presence of 5mM ATP (not shown). It seems that a degradative activity, not inhibited by ATP eluted out of the P-11 column slightly later than the 2'-5'A binding protein.

DISCUSSION

We have shown that 2'-5'A binds to a discrete number of proteins in mammalian extracts, one major cytoplasmic protein and four or five nuclear proteins (Fig 11). However, we showed that different extraction procedures of nuclei gave similar but not identical sets of 2'-5'A binding proteins. The physiological role, if any, of these proteins remains unknown. It is tempting to speculate that these proteins may be involved in anti-viral or anti-mitogenic mechnisms. On the other hand, a 2'-5'A dependent RNase L activity could be involved in RNA processing.

We present a scheme for the partial purification of the cytoplasmic 2'-5'A binding protein, the RNase L. Our purification scheme represents the continuation of work of our laboratory, which started with the discovery of the enzyme in 1976 (93).

The selection of calf spleens as starting material replaces the laborious and costly growth of cells in culture. Moreover calf spleens are a plentiful, accessible and generous source of RNase L. The relative abundance of RNase L in spleen and lymphoid tissues and cell lines was noted previously by others (122,123, G. Floyd-Smith, personal communication). We developed an extraction procedure , borrowing from several published purification attempts and

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following the general recommendations described by Strode (224).

Gentle extraction resulted in a cell lysate containing a single major 2'-5'A binding protein with m.w. of 78kD. Homogenization in the Virtis blade homogenizer at higher rpm (5-10,000), however, resulted in multiple binding proteins most probably representing degradation products of the 78 kD protein (143).

A 2'-5'A binding protein sedimented with the pellet of the S200 and migrated with a m.w. of 44kD in SDS-PAGE. The bulk of the 78 kD remained in the supernatant of the S200 (Fig. 13A). Baglioni and coworkers find most of the 2'-5'A binding activity in HeLa cells to be ribosome associated (122). On the other hand, we detect the majority of this activity in post-ribosomal supernatants. Baglioni did not identify the size(s) of the ribosome associated 2'-5'A binding protein in Hela cells (122).

The S200 contained a 2'-5'A dependent nuclease, however this activity could only be assayed when the extracts were diluted several thousand fold (Fig. 14D); indicating the presence of other nucleases in the preparation.

The bulk of the 2'-5'A degrading activity sedimented with the pellet fraction of the S200 as was described for HeLa cells (122); however the supernatant fraction still had a degradative activity (Fig. 13B).

The steps comprising the pre-affinity purification were aimed at reducing the amount of total protein and the volume of the extract and furthermore at purifying the 2'-5'A binding protein away from nucleases that degrade 2'-5'A. We presented two methods of

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pre-affinity purification. Most of the obvious conclusions were mentioned in the results section of this chapter, below we consider two findings.

The 2'-5'A binding activity eluted as two peaks from DEAE, which on crosslinking produced indistinguishable products (not shown), a similar finding was reported for the EAT cytoplasmic enzyme (125). The possibility exists that there are several RNase L isoenzymes with slightly different functions. Apparent redundancy is not rare in the IFN system , eg. more than 15 IFNs and at least four oligoadenylate synthetases . Two other findings suggest the possibility that there may be more than one enzyme: First, the K_a for the cytoplasmic 2'-5'A binding changed after IFN treatment of EAT cells (not shown). Second, when measuring the half-life of the cytoplasmic 2'-5'A binding activity in EAT cells we found that the bulk of this activity decayed very fast, however a certain fraction of the original measured activity decayed very slowly (not shown).

Activities that degraded (2'-5')pppApApApA[32P]pCp were never completely separated from the 2'-5'A binding activity. Fractions purified through phosphocellulose supplemented with either potassium phosphate or ATP, however, minimally degraded the 2'-5'A derivative (Fig. 16C). This makes it likely that the degradation required the presence of a phosphatase.

The development of an affinity column using (2'-5') pppApApApA as the ligand was delayed until the degradation of this oligonucleotide was controlled. We synthesized several affinity matrices. The first class utilized derivatives of 2'-5'A of the the following general formula: (2'-5') pppApApApA>NH-CH_2CH_2SH.

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These were reversibly linked to sulfhydryl containing matrices (230,231). These derivatives could be released from the solid matrix by washing with ßmercaptoethanol. Once incubated with the P-11 purified spleen extracts these 2'-5'A derivatives were spontaneously released from the matrices. The mechanism of this release was never precisely determined, however, disulfide exchanges with free SH groups in the matrices seemed probable.

We also synthesized affinity matrices by periodate oxidating (2'-5')pppApApApA and linking it to a hydrazide matrix (229). These matrices were shown to deplete spleen extracts of 2'-5'A binding activity. The 2'-5'A binding activity was never unambiguosly recovered. Studies at present are continuing a search for conditions or agents capable of eluting 2'-5'A binding activity from these matrices. One hopeful candidate is a 2'-5'A-iminobiotin derivative of 2'-5'A. This can be coupled to streptavidin in a reversible fashion (232).

Three major goals were seen as necessary to have a workable purification protocol: a good starting material, a pre-affinity purification scheme and an affinity column. Here I describe work on the first two and mentioned the early work on the third. The schemes described here coupled with an affinity column should yield homogenous RNase L.

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CHAPTER FIVE

A 2'-5'OLIGOADENYLATE BINDING PROTEIN IN PLANTS.

INTRODUCTION

The 2'-5'oligoadenylate synthetases, the RNase L, and the 2'-5' oligoadenylates have been implicated in mediating some of the anti-viral effects of interferons and may also be involved in the control of cell growth (22,66,81,83,84). Several studies have demonstrated the presence of ppp(A2'p)$_n$A and ppp(A2'p)$_n$A binding proteins in organisms other than mammals. ppp(A2'p)$_n$A or the core 2'-5'oligoadenylates ((A2'p)$_n$A or 2'-5'A) have been found in many organisms including E. coli, yeast and several higher vertebrates (124,226b). The presence of proteins with high affinity for ppp(A2'p)$_n$A has been demonstrated in mammalian tissues and cells in culture, in reptilian tissues, and in low levels in amphibian tissues (122,124).

The existence of a 2'-5'A system in plants is a subject of considerable controversy. Sela and coworkers and others have published several studies demonstrating an anti-viral effect of 2'-5'oligoadenylates when applied topically to tobacco plants prior to tobacco mosaic virus (TMV) infection (227,228). However, Cayley et

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al. could not reproduce Sela's results using a very similar system (124). ppp(A2'p)$_n$A-like oligonucleotides have been detected in tobacco plant extracts, but to our knowledge no ppp(A2'p)$_n$A binding activity has been found in these extracts (124,228b).

We undertook the following work to investigate whether wheat contains a detectable ppp(A2'p)$_n$A-binding activity.

MATERIALS AND METHODS

Wheat extracts. Whole cell extracts were prepared from fresh etiolated shoots of wheat (*Triticum aestivum* var sinton) germinated in the dark at 25°C. 5-7cm long shoots were harvested, cut into 1 cm pieces and immersed in 30mM Hepes (pH 7.9), 5mM $MgCl_2$, 0.5mM EDTA, 3mM dithiothreitol (DTT), 1mM phenylmethylsulfonylfluoride (PMSF), 12.5% sucrose (w/v), 25% glycerol (v/v). The shoot suspension was homogenized in a Waring Blender at full speed for 16 sec and then in a Virtis blade homogenizer at 45,000 rpm for three pulses of 16 seconds each. These and other procedures were performed at 0-4°C unless otherwise specified. The crude homogenate was filtered through sterile pre-wetted Miracloth (Calbiochem) and supplemented with 1/10 volume of 4M $(NH_4)_2SO_4$ (pH 7.9) added dropwise with stirring. After an additional 16 min of stirring the homogenate was spun in a Ti-70 rotor (Beckman) at 50,000 rpm for 2.5 hr . The resulting clear, tan supernatant was dialyzed against 2 x 1L volumes of 20mM Hepes (pH 7.9), 75mM $(NH_4)_2SO_4$, 1mM DTT, 0.5mM PMSF, 0.2mM EDTA, 15% glycerol (v/v) for a total of 12 hr. Aliquots were frozen in liquid nitrogen and stored at -80°C. Wheat embryos were isolated

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(222) and extracts made as described for the shoots. Each aliquot was thawed immediately before use in the binding assays supplemented with the following protease inhibitors: leupeptin (16 μg/ml), aprotinin (5 μg/ml), 1,10-phenanthroline (1mM) , benzamidine (1 mM), sodium metabisulfite (1 mM), soybean trypsin inhibitor (50 μg/ml) and ovomucoid trypsin inhibitor (50 μg/ml) as described by Wells et al (223).

Binding assays. All the binding assays were done by incubating 10μl of 10X binding buffer (250 mM Tris-HCl (pH 7.4), 30mM ß-mercaptoethanol, 10mM MgAcetate), the indicated amount of (p)pp(A2'p)$_3$A[^{32}P]pCp (Amersham) in 20mM potassium phosphate buffer (pH 7.0), the indicated amount of wheat extract, and distilled water to a volume of 100 μl. After one hour the reaction mixture was filtered through nitrocellulose (Millipore, pore size = 45 μ) filters (114) pre-soaked in wash buffer (25mM potassium phosphate (pH 7.4), 50mM KCl, 30mM ßMeOH , 5mM Mg Acetate) supplemented with NaPPi to 4% (w/v). The filters were then washed with 5 ml of wash buffer .

Crosslinking assays. The wheat shoot extracts were incubated with (2'-5')pppApApApA[^{32}P]pCp under the conditions described for the binding assay and irradiated with UV-light as described by Floyd-Smith et al (119). Crosslinked products were fractionated in a SDS- 10% polyacrylamide gel (201), and visualized by autoradiography on XAR-5 film (Kodak) with one intensifying screen at -70° C for 72 hours.

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RESULTS

Whole cell extracts from wheat embryos and shoots were found to have a (2'-5')pppApApApA[^{32}P]pCp binding activity. The binding was shown to increase linearly with the amount of wheat shoot extract added to the binding assay (Fig. 17A). The binding reached a saturation level even in the presence of excess input (2'-5')pppApApApA[^{32}P]pCp, this was also observed with mammalian extracts by others (122) and by us (data not shown). The binding activity in wheat shoot extracts saturated at concentrations of (2'-5')pppApApApA[^{32}P]pCp over 5.0 x 10^{-10} M, with the curve predicting a K_d of 4.5 x 10^{-10} M , (Fig. 17B) 10^{-8} M ppp(A2'p)$_3$A decreased the binding of (2'-5')pppApApApA[^{32}P]pCp in wheat shoot extracts to below 50% of the binding without inhibitor, 10^{-7} M completely inhibited the binding while 10^{-3} M ATP was required to obtain the same inhibition, (Fig. 17C). 10^{-3} M pCp had no effect on the binding of (2'-5')pppApApApA[^{32}P]pCp, (not shown), indicating that the bulk of the labeled ligand in the binding assays could not have been free [^{32}P]pCp. (A2'p)$_3$A (core) and (A3'p)$_3$A added at concentrations as high as 100μM did not compete with the binding of (2'-5')pppApApApA[^{32}P]pCp (data not shown). Unfortunately ppp(A3'p)$_3$A was not available to use in competition experiments.

The wheat shoot binding activity was labile; it was abolished by incubation at 37°C for 15 min. The activity also disappeared upon treatment with proteinase K at 4°C for 1 h. In the absence of proteinase K the activity was stable at 4°C for over one hour (data not shown). These findings make it likely that the

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Figure 17. A (2'-5')pppApApApA[32P]pCp binding activity in wheat shoot extracts. A. Extract dose dependence of the binding. Binding assays with 9.9×10^{-10}M (2'-5')pppApApApA[^{32}P]pCp and varying amounts of extract , 5 to 40 µl, added per assay. The extract used was wheat shoot extract prepared as described above, containing 0.7 mg protein per ml. Nitrocellulose filters were counted in a Nuclear Chicago scintillation counter, without scintillation fluid; retained cpms are plotted (-•-) vs. volume of extract added (µl). The points correspond to the following added extract volumes: none, 5µl, 10µl, 25µl and 40µl. **B.** (2'-5')pppApApApA[^{32}P]pCp dose dependence of the binding. Binding assays using 25 µl of wheat shoot extracts and varying concentrations of (2'-5')pppApApApA[^{32}P]pCp (from 3.3×10^{-11}M to 9.9×10^{-10}M). Retained cpms are plotted (-•-) in a semi-log graph vs. concentration of added (2'-5')pppApApApA[^{32}P]pCp. The points correspond to the following concentrations of (2'-5')pppApApApA[^{32}P]pCp in the reaction mixtures: 3.3×10^{-11} M, 1.67×10^{-10} M, 3.3×10^{-10} M, 4.95×10^{-10} M, 9.9×10^{-10} M. **C.** Inhibition of binding by excess unlabeled ppp(A2'p)$_3$A. Binding assays with 5×10^{-10}M (2'-5')pppApApApA[^{32}P]pCp, 40 µl of wheat extract and supplemented with inhibitors (**I**) of the binding; either ppp(A2'p)$_3$A, pCp or ATP. Binding (%) was calculated using the following formula: Binding = [(Retained cpms in the presence of **I**) x 100]/ (retained cpms in the absence of **I**). This was plotted vs. the -log[**I**], for **I** = ppp(A2'p)$_3$A, (-•-) and ATP, (-Δ-); The binding in the absence of inhibitors (**C**), when [**I**] = 0 and therefore -log[**I**] = ∞, is defined as 100%. **D.** Crosslinking of (2'-5')pppApApApA[32P]pCp to a 67,000 dalton protein in wheat extracts. A.Crosslinking of a 67,000 dalton protein in wheat shoots. Binding assays contained 9×10^{-10} M (2'-5')pppApApApA[^{32}P]pCp and 25µl of wheat extract. Lane 1, molecular size markers, identified by the dots; carbonic anhydrase, 30,000 daltons; ovalbumin, 43,000 daltons; bovine serum albumin, 68,000 daltons and phosphorylase b, 92,500 daltons. Crosslinked products from wheat embryo extracts, approximate molecular weights 60,000 (**a**) and 67,000 (**b**) , lane 2; crosslinked products from wheat shoot extracts, approximate molecular weight 67,000 (**b**), lane 3; crosslinked products from cytoplasmic extracts of mouse EAT cells, approximate molecular weight 78,000 (**L**), lane 4. **E.** Inhibition of crosslinking of the

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67,000 dalton protein (arrow) by excess unlabeled ppp(A2'p)3A. Binding assays as above except supplemented with unlabeled ppp(A2'p)$_3$A to the following concentrations: none added, lane 1; 10^{-12}M, lane 2; 10^{-11}M, lane 3; 10^{-10}M, lane 4; 10^{-9}M, lane 5; 10^{-8}M, lane 6; 10^{-7}M, lane 7.

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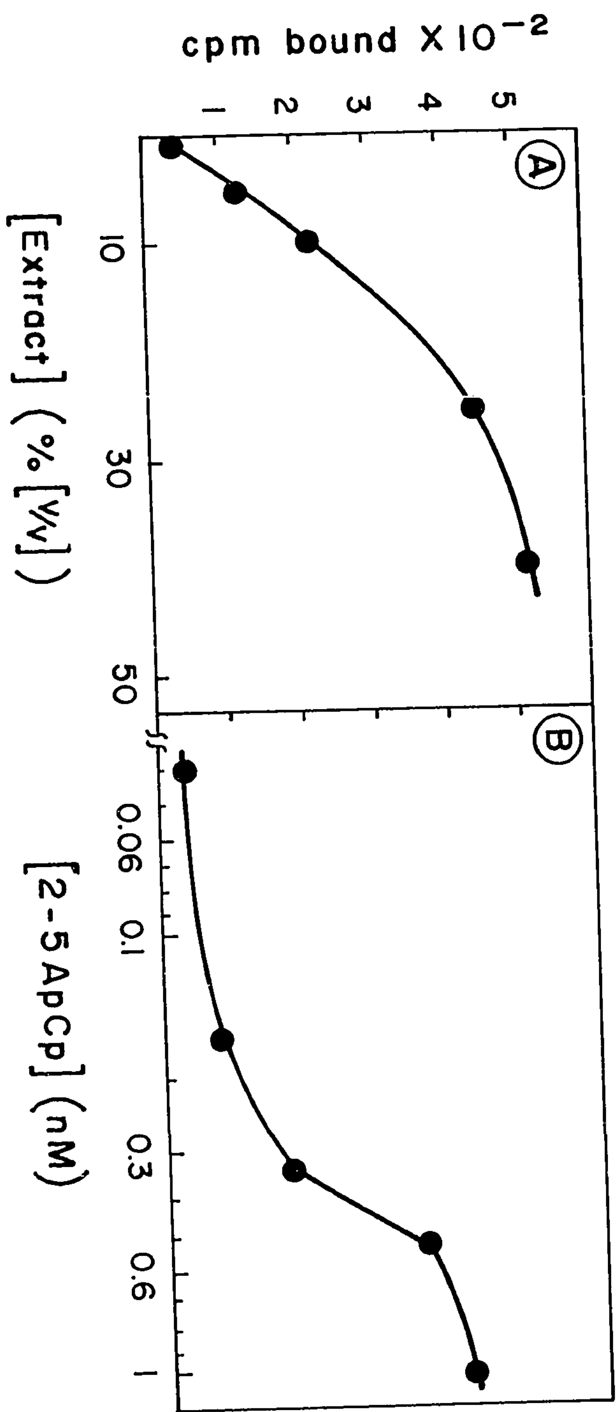

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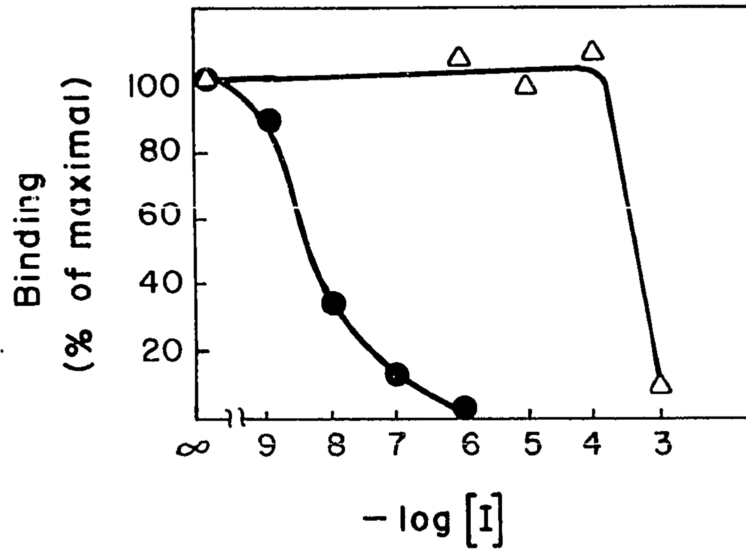

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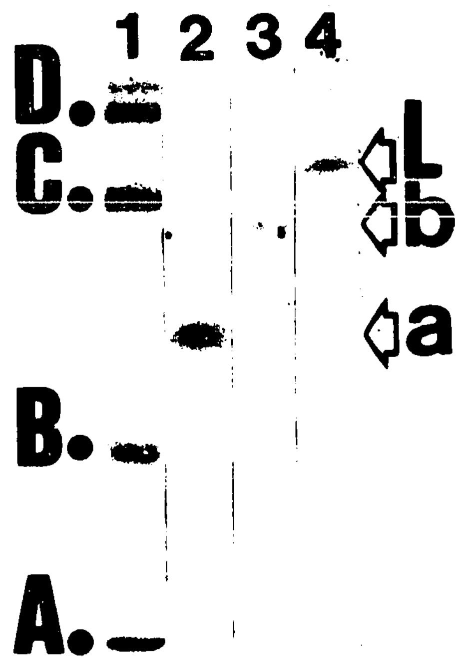

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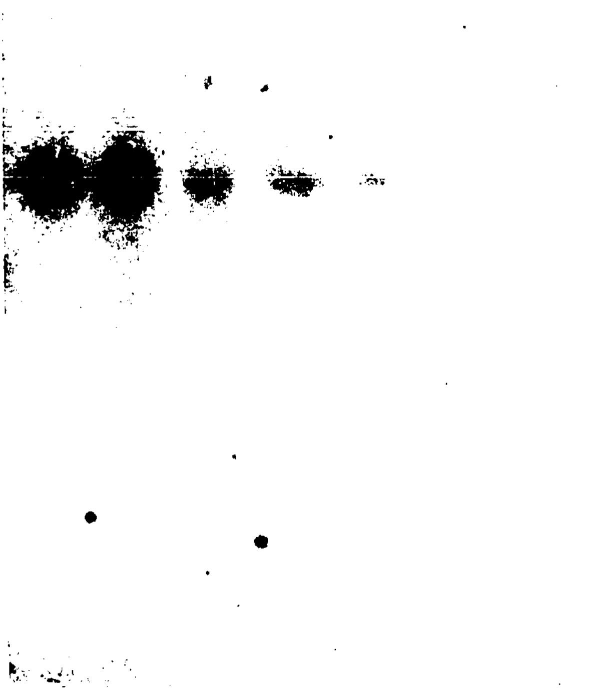

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activity resides in a protein, hereinafter we will refer to the binding activity as a binding protein .

To characterize the molecular weight of the binding protein we used the UV-crosslinking method described by Floyd-Smith et al (119). This procedure resulted in the crosslinking of (2'-5') pppApApApA[^{32}P]pCp to a protein that migrated in SDS-PAGE with an apparent molecular weight of 67,000 daltons (Fig 17D, lane 3). Cytoplasmic extracts of Ehrlich ascites tumor cells treated in a similar way contain a single crosslinked product with an apparent molecular weight of 78,000 daltons (Fig 17D, lane 4). The crosslinking of (2'-5')pppApApApA[^{32}P]pCp to the 67,000 dalton wheat shoot protein was inhibited by addition of excess unlabeled ppp(A2'p)$_3$A to the binding reaction, (Fig 17E). The fact that only one protein was crosslinked to (2'-5')pppApApApA[^{32}P]pCp in wheat shoot extracts suggests that all of the detected binding activity can be accounted for by the crosslinked protein. The mol. weight of this crosslinked product is different from that of any ppp(A2'p)$_n$A binding protein reported in mammalian extracts.

In wheat embryo extracts two products (60,000 and 67,000 daltons) were crosslinked to (2'-5')pppApApApA[^{32}P]pCp (Fig. 17D, lane 2). Competition studies showed that the crosslinking to the 60,000 dalton product, which was the bulk of the crosslinked material, was not inhibited by addition of ppp(A2'p)$_3$A (not shown).

DISCUSSION

In this study we present evidence for the existence of a 67,000 dalton protein in wheat shoot extracts with high affinity for

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ppp(A2'p)$_3$A and for its labeled derivative (2'-5') pppApApApA[^{32}P]pCp. Others have attempted to find (2'-5') pppApApApA[^{32}P]pCp-binding proteins in higher plants and have not succeeded (124,228b). Some possible reasons for this are the following: Previous studies have used tobacco leaves or protoplasts; what we have detected may be a wheat specific , or a tissue specific protein. All previously studied extracts were exclusively cytoplasmic, ours was a whole cell extract. The relatively high abundance of phenolic compounds, quinones and endogenous proteases in plant tissues makes detection of labile activities in crude extracts difficult. We added many protease inhibitors

The binding of 2'-5' A to the 67,000 dalton protein in wheat extracts most probably represents a very specific interaction: as suggested by the very low dissociation constant of 4.5 x 10^{-10} M. Furthermore, the requirement for a phosphorylated 5' end indicates specificity akin to the mammalian RNase L. However, we cannot exclude the posibility that this protein has equal affinity for 3'-5' and 2'-5' linkages. Moreover, we do not know whether this protein preferrentially binds oligoadenylates over oligonucleotides containing other bases. The fact that only one protein in the crude wheat extract was UV-crosslinked to (2'-5')pppApApApA[^{32}P]pCp suggests another level of specificity.

The activity of this 67,000 dalton (2'-5') pppApApApA[^{32}P]pCp -binding protein in wheat shoots and embryos may or may not be related to the activity of the RNase L in mammalian cells. The level of endogenous RNases in these wheat extracts was very high (including enzymes which slowly degraded

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(2'-5')pppApApApA[^{32}P]pCp, making affinity chromatography with 2'-5'oligoadenylate columns difficult in these unfractionated extracts) preventing the detection of a ppp(A2'p)$_3$A dependent RNase activity (data not shown). It remains to be established if the activity described here plays a role in an anti-viral and/or growth control system in plants. If this is a protein related to the RNase L in mammals, then the 2'-5' A system may represent a very early development in evolution.

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CHAPTER SIX

REGULATION OF THE 2'-5' A SYSTEM IN BALB/C-3T3 CELLS BY PLATELET-DERIVED GROWTH FACTOR.

INTRODUCTION

Interferons (IFNs) were first discovered because of their potent anti-viral action. IFNs have many other actions, including an anti-proliferative effect on cells (26). A biochemical circuit, known as the 2'-5' A system, is regulated by IFNs. The IFN inducible 2'-5' A synthetases, the 2'-5' phosphodiesterase, RNase L, and 2'-5' A ((2'-5')oligoadenylates) compose the 2'-5' A system (22). In the presence of dsRNA the 2'-5' A synthetases convert ATP into 2'-5' A and PPi. 2'-5' A binds and activates the latent endonuclease, RNase L (22). The 2'-5' A phosphodiesterase controls the level of 2'-5' A and thus, negatively regulates the activity of the system (22).

The polypeptide growth factors are hormone-like proteins that are required for the growth of cells. These factors stimulate the growth of cycling cells and also induce the transition from quiescence to growth. The murine fibroblast cell line Balb/c-3T3 is dependent on the presence of Platelet-derived growth factor (PDGF) in order to proliferate (236-239b). In the absence of PDGF these

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cells become quiescent (237). Quiescent Balb/c-3T3 cells exposed to PDGF will go through a G0->G1 transition and are said to be competent to divide (237). Further progression through the cell cycle is dependent on other factors present in serum (239).

IFNs inhibit the growth of cells in culture and in animals (1), and have been postulated to have a physiological role in the regulation of normal cell growth (1,26,34,30). IFNs slow the growth of cycling cells (26,26b, 26c) and also impair the transition from the quiescent state (G0) to DNA synthesis and cell division mediated by growth factors (9,13, 63,195).

The 2'-5' A system may mediate some of the anti-proliferative action of IFN (1,10,22,78,87,88,141-145). It is clear that changes in the proliferative state of cells correlate with changes in the level of 2'-5' A synthetase; however, the direction of these changes is not uniform. The level of a 2'-5' A synthetase was shown to be higher in non-proliferating cells than in proliferating lymphoid cells (86-88). On the other hand, there is a lack of correlation between cell growth and levels of 2'-5' A synthetases in lymphoblastoid cell lines when growth was inhibited by retinoic acid (234). Mallucci and coworkers have shown a marked induction of a synthetase in mouse embryo fibroblasts traversing the S phase of the cell cycle (233). Growth factors were shown to induce the expression of 2'-5' A synthetases or their transcripts. Epidermal growth factor increases the level of a 2'-5' A synthetase and stimulates growth of quiescent human fibroblasts (89). Recently Zullo et al showed that PDGF induces the expression of a 1.8 kb transcript that hybridizes with a human 2'-5' A synthetase

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oligonucleotide probe in quiescent Balb/c-3T3 cells (13). Moreover, nerve growth factor (NGF), albeit not a mitogen, was shown to increase the level of both a 2'-5' A synthetase and 2'-5' A in rat pheochromocytoma PC-12 cells (235).

The level of the RNase L is also regulated during changes in cell growth state, but this has not been studied extensively. Silverman and co-workers showed that as JLS-V9R and NIH-3T3 mouse cells approach confluence the levels of the cytoplasmic 2'-5'A binding protein, RNase L, increase (141-143). This increase is not mediated by secretion of a known IFN (141-143). Differentiated embryonal carcinoma cell lines have detectable levels of RNase L, whereas undifferentiated carcinoma cell lines do not (144). Furthermore, when an undifferentiated cell line (PC13) differentiates in culture the expression of RNase L is induced (144).

Tominaga and Lengyel showed that when Balb/c-3T3 cells were treated with murine ß-IFN for 48h prior to PDGF addition the mitogenic response was abrogated whereas the induction of c-myc RNA was unaffected (63). On the other hand Einat et al reported that concomitant addition of a murine IFN preparation and PDGF inhibited both the mitogenic response and the induction of c-myc RNA. Moreover, in a related series of reports it has been clearly shown that cycling Daudi cells treated with IFN have much lower levels of c-myc transcripts than untreated cells (196-198). These cells are very sensitive to the anti-proliferative effect of IFN (196-198).

We studied the regulation of the 2'-5' A system by PDGF. We used Balb/c-3T3 cells because its growth is controlled in

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culture in ways that resemble normal growth control in normal tissues. In this chapter I show the following: 1) Partially purified PDGF induced the expression of two 2'-5' A synthetase transcripts and of 2'-5' A synthetase activity. 2) Recombinant PDGF and calf serum had the same effects. 3) Partially purified PDGF decreased the level of RNase L. 4) ßIFN inhibited the mitogenic stimulus of PDGF without affecting the induction of c-myc RNA in confluent Balb/c-3T3 cells.

MATERIALS AND METHODS

Interferons and growth factors. Electrophoretically pure mouse ß IFN (specific activity , 109 NIH mouse interferon reference units per mg protein) was produced and purified as previously described (199). IFN doses will be given in NIH mouse interferon reference standard units/ml (U/ml). Platelet extract (partially purified PDGF) containing 10,000 units of PDGF per ml was a boiled extract from freshly isolated human platelets, prepared as described in ref (237). PDGF (B-chain homodimer) purified from v-sis transformed cells was purchased from Amgen, Inc. TGFß was a gift of Genentech, Inc. and EGF was a gift of Dr. J. Rheinwald, Dana Farber Cancer Institute.

Cells and growth conditions. Balb/c-3T3 cells (clone A31) were grown as monolayers in DMEM supplemented with 10% calf serum at 37°C in a 5% CO_2 atmosphere. The medium was exchanged every 72 h until cells were confluent at which time they were passaged. To obtain quiescent monolayers, the cells were passaged 1:5 on day 0, and the media exchanged on day 3. On day 6

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the cells were washed in DMEM without serum or PBS and grown in DMEM supplemented with 5% of human platelet poor plasma (PPP) for 24 h unless stated otherwise. At this time, the cells were quiescent as documented by [3H]-thymidine autoradiography (237). 300 U/ml of PDGF was added unless otherwise stated; this dose of PDGF induced DNA synthesis in 100% of the cells.

Cell counting. Cells were washed twice with PBS, exposed to trypsin for 10 sec and incubated at 37°C for 5 min. Cells were resuspended and counted in a Coulter Counter.

Cytoplasmic extracts. Cells in monolayer culture were washed with chilled PBS and scraped with a rubber policeman. The cells were spun at 1000 g at 4 °C for 5 min and washed in 10 ml of chilled PBS. The cell pellet was resuspended in 1ml of PBS and transferred to 1.5 ml Eppendorf tubes, spun at 12,000g at 4 °C for 15 secs. The cell pellet was resuspended in 2 packed cell volumes of lysis buffer (20mM Hepes-KOH (pH 7.4),10mM KCl, 1. 5mM $Mg(OAc)_2$, and 15mM ß-mercaptoethanol) supplemented with protease inhibitors as described in ref 223. Nonidet P-40 (NP-40) was added to the cell suspension to a concentration of 0.5% (w/v), and this was incubated for 10 min with vigorous vortexing every min. The celll nuclei were sedimented at 12,000g at 4 °C for 2 min and the supernatant fraction was collected and frozen in aliquots at -80°C.

2'-5' A synthetase assays. The solution assay. This 2'-5' A synthetase assay is a modification of the method of Nilsen et al (122). Cytoplasmic extracts (10-20 µg of protein) were incubated in 2'-5' A synthetase buffer (20mM Hepes-KOH (pH 7.4), 120mM KOAc, 25mM $Mg(OAc)_2$, and 10mM ß-MeOH)

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supplemented with 5mM ATP, 0.1μCi of alpha[32P]ATP (spec. act. 450Ci/mmol), and 100μg/ml polyI:polyC (Pharmacia) in a final volume of 25μl. The concentration of polyI:polyC was high in order to detect the enzymes with lower affinity for dsRNA (240). The samples were incubated at 30°C for 20h, heat inactivated at 95°C for 3 min, spun at 12,000g at room temperature for 5 sec, and incubated with 50mM $Mg(OAc)_2$, 12mM glucose, and 1.25 mg/ml of hexokinase (Sigma), at 30°C for 30 min. Hexokinase converts any remaining ATP to ADP, which can be separated easily from 2'-5' A in the TLC systems described below.

2'-5' A synthetase assays. The polyI:polyC agarose assay. Cellular extracts were incubated with an equal volume of polyI:polyC agarose (Pharmacia) suspension (1:1) in 2'-5' A synthetase buffer. These were incubated at 4°C for 8 h with end over end rotation, spun at 12,000 g at 4°C for 3 min and the supernatant fraction was discarded. The pellet fraction was washed once with 150 μl of 2'-5' A synthetase buffer and incubated in 10 μl of this buffer supplemented with ATP and alpha[32P]ATP as in the solution assay. After heat inactivation the samples were spun at 12,000 g at RT for 3 min and 5 μl of the supernatant fraction was digested with hexokinase as described above.

Thin layer chromatography. We fractionated the nucleotides present in the reaction mixtures using TLC. Upon completion of the hexokinase reactions we spun these at 12,000 g at RT for 25 sec, and spotted 1 μl of the supernatant fraction on PEI-cellulose thin layer chromatography plates (Sigma). The chromatographs were developed with 0.75 M potassium phosphate

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(pH 3.7) or 0.25 M ammonium bicarbonate. Products were visualized by autoradiography.

[35S]methionine labeling, TCA precipitation and SDS-PAGE. [35S]methionine labeling, TCA precipitation and SDS-PAGE were done as described in Garcia-Blanco et al (240b).

Extraction of total cellular RNA. Cells were washed with autoclaved PBS and lysed by addition of 4M Guanidinium isothiocyanate, 25 mM NaOAc, 100 mM ßMeOH (248). The viscous lysates were collected by scraping with a rubber policeman and gently pipetting into a 15 ml polystyrene tube. The lysates were frozen at -20°C and stored for no longer than one week. The lysates were quickly thawed and the DNA was sheared by vigorously vortexing for 1 min followed by 10 passes through a 20 gauge needle. Three mls of sheared lysates were gently layered over 2 mls of 5.7 M CsCl, 25 mM NaCitrate cushion, and spun in a SW50.1 rotor (Beckman) at 40,000 rpm at 20°C for 16 h. The RNA pellet fractions were resuspended in water, supplemented to 0.3 M NaOAc (pH 5.5) and EtOH precipitated. The RNA precipitates were resuspended in water and stored at -80 °C.

Analysis of RNA products. Total cellular RNA was fractionated in formamide-formaldehyde agarose gels and transferred to nitrocellulose paper essentially as described (213). A murine 2'-5' A synthetase cDNA (M2), kindly donated by B.R.G. Williams (Hospital for Sick Children, Toronto) was used as a probe (239c) to detect 2'-5' A synthetase transcripts on the blots. DNA was nick-trasnslated by the method of Rigby (214).

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RESULTS

Partially purified PDGF induces the expression of 2'-5' A synthetase transcripts and of a 2'-5' A synthetase in quiescent Balb/c-3T3 cells . Confluent, quiescent Balb/c-3T3 cells were treated with 500 U/ml of mouse ßIFN or 300 U/ml of partially purified PDGF for varying lengths of time. Total RNA was extracted and 2'-5' A synthetase transcripts were detected using Northern blot analysis. The probe used was derived from a cDNA clone made from RNA extracted from interferon treated mouse JLSV-9R cells (239c) The blots probed with the nick-translated 2'-5' A synthetase cDNA were washed at high stringency. Murine ßIFN and partially purified PDGF induced the expression of transcripts of similar, if not identical, size (Fig 18). One of this transcripts was of similar size to an IFN-inducible 1.8 kb 2'-5' A synthetase transcript in JLSV-9R cells (239c). Hereinafter we will refer to this 2'-5' A synthetase transcript as the 1.8 kb transcript, albeit we did not rigorously establish its precise length. The larger transcript was approximately 3.6 to 4 kb in length, and will be referred to as the 4 kb transcript.

Both IFN and partially purified PDGF induced accumulation of the 4 kb transcript quickly and transiently; with maximal levels reached by 3 h in both cases (Fig 18). Both IFN and partially purified PDGF induced accumulation of the 1.8 kb with slower kinetics (Fig 18). IFN was a better inducer of the expression of the 4 kb transcript than of the 1.8 kb transcript, whereas, PDGF was a better inducer of the 1.8 kb transcript than of the 4.0 kb transcript. The level of the 1.8 kb transcript that hybridized with this probe

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Figure 18. IFN and Partially Purified PDGF Induce the Expression of 2'-5' A Synthetase RNAs. Time Course of Inducton. Quiescent Balb/c-3T3 cells were treated with 500 U/ml of mouse ßIFN (panel A) or 300 U/ml of partially purified PDGF (panel B) for varying lengths of time. Total RNA was extracted, purified, 5 μg of this was fractionated and transferred to nitrocellulose paper. 2'-5' A synthetase transcripts were detected by hybridizatrion of the immobilized RNA to a radiolabeled murine 2'-5' A synthetase cDNA probe and visualized by autoradiography. The level of the 1.8 kb (-•-) and ~ 4 kb (-o-) 2'-5' A synthetase transcripts is plotted vs. time of exposure to either mouse ßIFN or partially purified PDGF. Quantification of the autoradiographic signal corresponding to the 2'-5' A synthetase RNAs was obtained with an LKB gel scanner. Insets show actual autoradiographic pattern.

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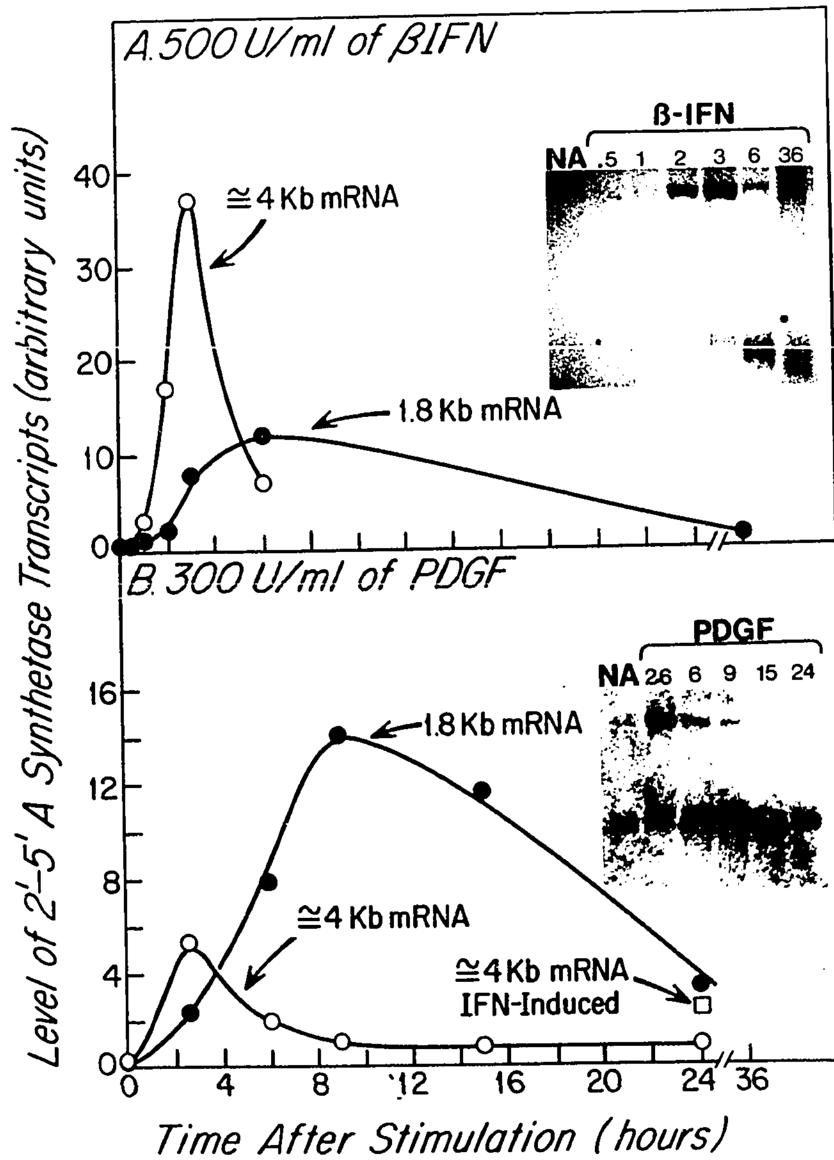

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was induced 3-to 4-fold as early as 160 min after stimulation with partially purified PDGF (Fig 18, panel B). The induction was maximal, 13-fold, at 9 h and diminished to almost background by 24 h post partially purified PDGF treatment (Fig 18, panel B).

The induction of the 1.8kb and 4 kb transcripts was dependent on the dose of partially purified PDGF added to the cells (not shown). 30 U/ml, equivalent to 6 ng/ml of purified PDGF, was the lowest dose that resulted in a detectable response (2-fold), and 120 U/ml resulted in almost maximal induction of both 2'-5' A synthetase transcripts (not shown).

Balb/c-3T3 cells were grown to confluence and growth arrested in DMEM supplemented with 5% PPP for 24 h. The cells were treated with 300 U/ml of partially purified PDGF and cell lysates were made 2, 8,12 and 24 h hence. The cytoplasmic 2'-5' A synthetase activity was measured using the solution assay. The 2'-5'A made was visualized by autoradiography of TLC plates and quantified by scanning on a soft laser scanner. This method was shown to be equivalent to cutting and counting of TLC plates. The average of the 2'-5' A synthetase activities present in extracts made from duplicate cell cultures is shown (Fig 19). The synthetase activity increased by 3-fold within 2 h and by 8 h it had increased over 10-fold (Fig 19). Furthermore, the activity remained elevated for up to 24 h after partially purified PDGF addition (Fig 19). This result was also obtained using the polyI:polyC agarose assay described. The cytoplasmic extracts were incubated with polyI:polyC agarose beads, and the bound 2'-5'A synthetase was assayed. This procedure reduces the probability that a 2'-5'

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Figure 19. Partially Purified PDGF Induces the Expression of a 2'-5' A Synthetase in Quiescent Balb/c-3T3 cells: Time Course of Induction. Quiescent Balb/c-3T3 cells were treated with 300 U/ml of partially purified PDGF for varying lengths of time, cytoplasmic extracts were made and assayed for 2'-5' A synthetase activity by the solution assay described in the materials and methods. The relative amount of 2'-5'A synthesized, a measure of 2'-5' A synthetase activity in the absence (dotted bars) or the presence (hatched bars) of 100μg/ml of polyI:polyC is plotted vs. time of exposure to partially purified PDGF.

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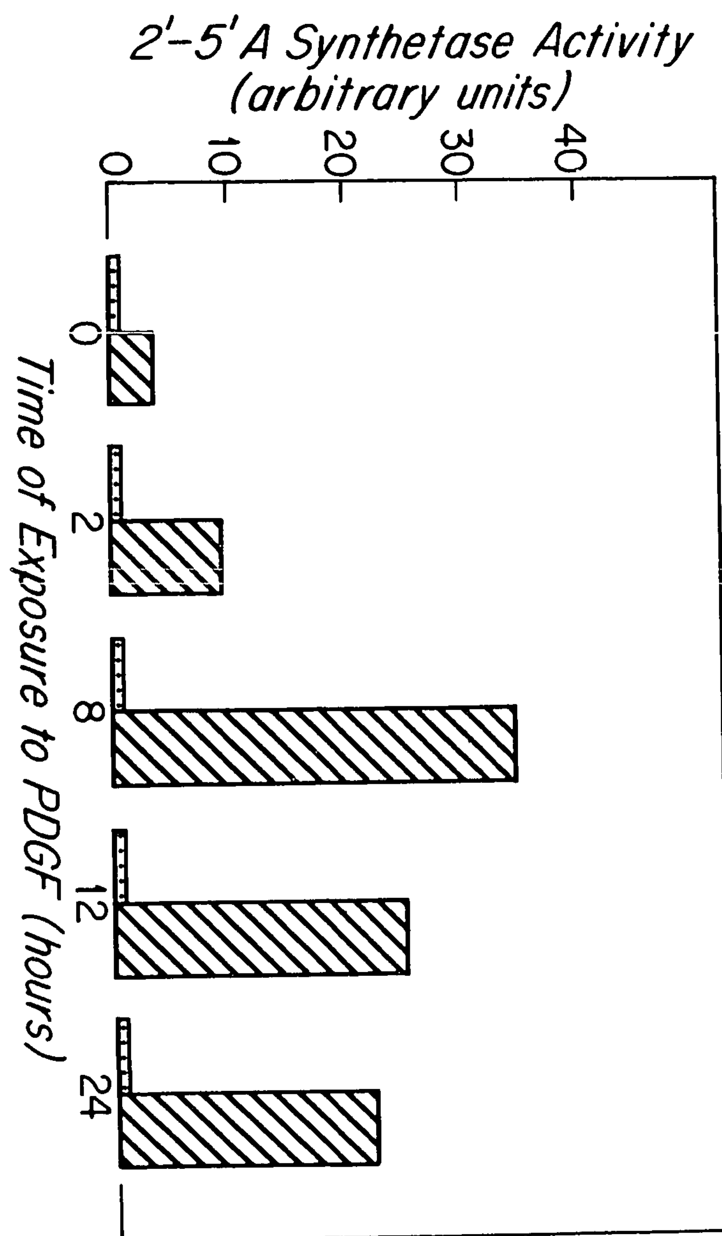

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phosphodiesterase would interfere with the results of the assay. A typical result is shown in Fig 21, and will be discussed below.

Purified PDGF induces the expression of a 2'-5' A synthetase RNA and of a 2'-5' A synthetase. In order to determine whether pure PDGF would induce a 2'-5' A synthetase, we treated quiescent cells with purified PDGF. This PDGF, a B-chain homodimer, is encoded by the v-sis gene of simian sarcoma virus. Purified PDGF induced the expression of a 1.8 kb 2'-5' A synthetase transcript in confluent,quiescent Balb/c-3T3 cells (Fig 20). Quiescent cells were stimulated with PDGF for 9 h and the RNA was extracted. The induction was dependent on the dose added to the cells, and was detectable at 12.5 ng/ml of PDGF (data not shown). At a concentration of 20 ng/ml, PDGF increased the level of the 1.8 kb RNA 5.4-fold.

We tested whether or not other growth factors known, or suspected, to be active in the boiled platelet extract were contributing to the induction of 2'-5' A synthetase transcripts seen with this impure PDGF preparation. Neither 20 ng/ml of TGFß (Fig 20) or 5 µg/ml of insulin (not shown) induced a 2'-5' A synthetase transcript . 10 ng/ml of EGF had a modest effect, 2.4 fold induction, on the level of the 1.8 kb RNA (Fig 20). Only the combinations of growth factors that contained PDGF resulted in a marked induction of the 1.8 kb 2'-5' A synthetase transcript (Fig 20)

We also tested whether or not these factors would enhance the response to PDGF if added in combination with it. The effect of the other growth factors (EGF and TGFß) on the PDGF induction of the 1.8 kb 2'-5' A synthetase transcript was modest (Fig 20). Induction

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Figure 20. PDGF Induces the Expression of a 1.8 kb 2'-5' A Synthetase RNA in Quiescent Balb/c-3T3 cells. Quiescent Balb/c-3T3 cells were treated for 8 h with the indicated growth factor(s), otherwise as described in figure 1. The dose of purified PDGF (B chain homodimer) was 20 ng/ml, EGF was 10 ng/ml and TGFß was 50 ng/ml. The level of the 1.8 kb 2'-5' A synthetase transcript RNA is plotted vs. agent(s) used to stimulate the cells.

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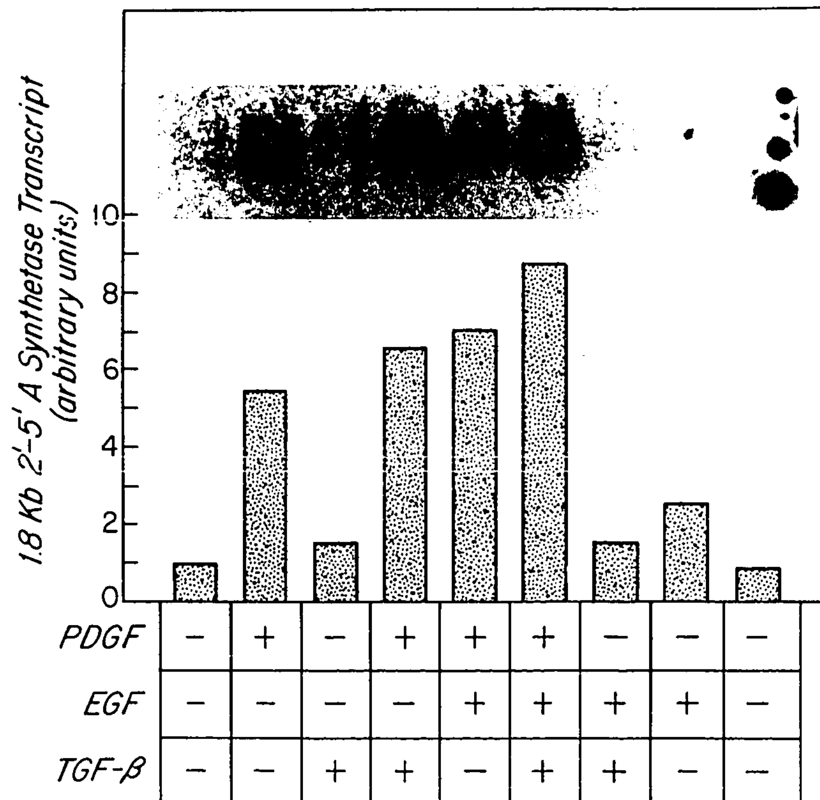

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of the JE gene, a competence gene strongly induced by pure PDGF, followed a very similar pattern of growth factor dependence (not shown).

We used the polyI:polyC agarose assay to determine the level of 2'-5' A synthetases in the cytoplasmic extracts. 2 ng/ml of PDGF modestly induced (3.9-fold) the 2'-5' A synthetase detectable in these extracts (Fig 21), whereas 10 ng/ml resulted in a strong induction (9.4-fold) (Fig 21). The induction of 2'-5' A synthetase by 500 U/ml of β IFN (20.2 fold) and by 300 U/ml of partially purified PDGF (10.1 fold) is shown for comparison (Fig 21)

Bovine calf serum induces the expression of 2'-5' A synthetase transcripts and of a 2'-5' A synthetase . Bovine calf serum contains PDGF and can induce quiescent Balb/c-3T3 cells to proliferate. Quiescent Balb/c-3T3 cells were treated with increasing doses of calf serum for 8 h and total RNA was extracted, purified and analyzed as described above. 10 % calf serum modestly induced accumulation of the 2'-5' A synthetase transcripts and 40 % calf serum strongly induced the accumulation of these RNAs (Fig 22). This transcript was of similar,if not identical, size as the 1.8 kb transcript and the kinetics of its induction were similar to those seen with partially purified PDGF (data not shown).

We investigated whether or not this calf serum would also regulate the levels of the 2'-5' A synthetase. Quiescent Balb/c-3T3 cells were exposed to 20% calf serum for varying lengths of time. Levels of 2'-5' A synthetase in cytoplasmic extracts from these cells were measured by the polyI:polyC agarose assay. Exposure to 20% calf serum caused an increase in the level of the 2'-

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Figure 21. PDGF Induces the Expression of a 2'-5' A Synthetase in Quiescent Balb/c-3T3 Cells. Quiescent Balb/c-3T3 cells were treated for 9 h with partially purified PDGF (PDGF), recombinant purified PDGF (rPDGF) or murine ßIFN at the doses indicated in the figure. Cytoplasmic 2'-5' A synthetase was assayed using the polyI:polyC agarose method described in the materials and methods. The relative amount of 2'-5'A synthesized, a measure of 2'-5' A synthetase activity, is plotted vs. the agent used to induce the cells. Inset shows the autoradiographic pattern of the TLC separations. Products of the synthetase assay were fractionated by TLC on PEI-cellulose plates using 0.250 M ammonium bicarbonate and visualized by autoradiography. 2'-5' A (**a**,**b**- most probably (2'-5')pppApApApA and (2'-5')pppApApA) and ADP (**c**) are identified.

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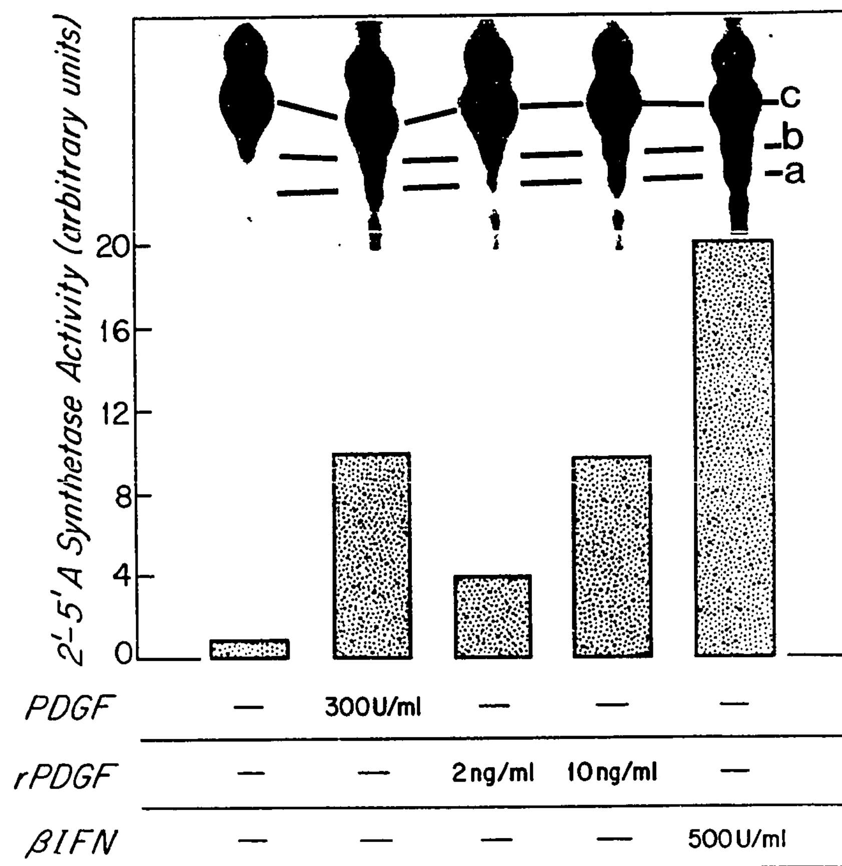

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Figure 22. Bovine Calf Serum Induces the Accumulation of a 1.8 kb 2'-5' A Synthetase RNA: A Dose Response. Quiescent Balb/c-3T3 cells were treated with various doses of calf serum for 8 h. 2'-5' A synthetase transcripts were detected as described in figure 1 and in the materials and methods. The level of the autoradiographic signals corresponding to the 1.8 kb RNA is plotted vs dose of calf serum. Inset shows the autoradiographic pattern.

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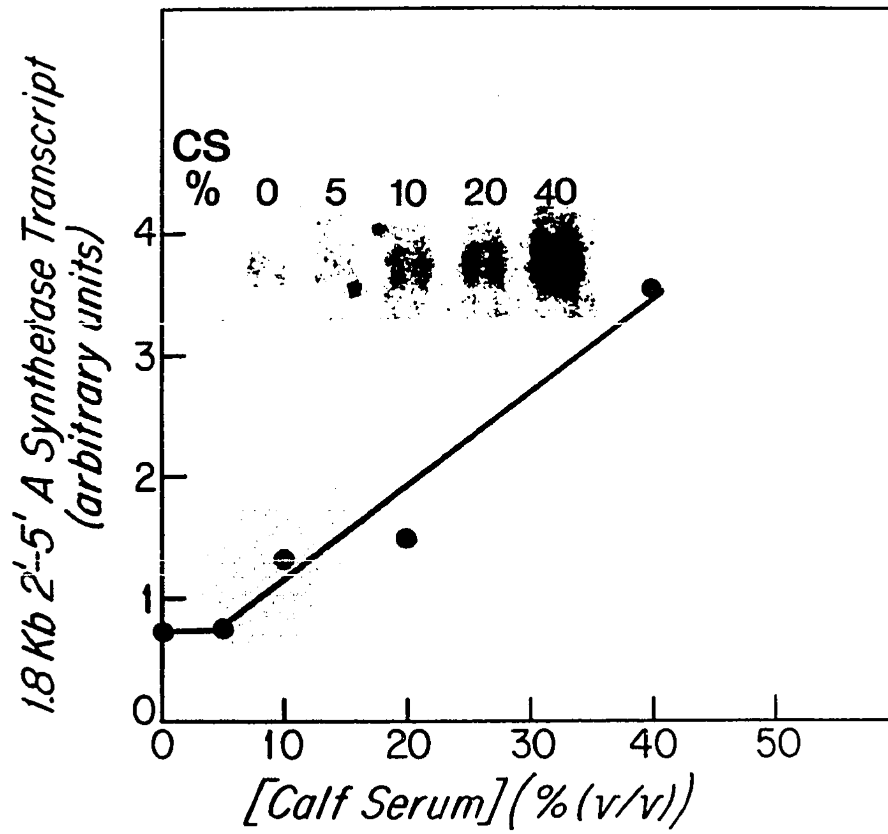

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5' A synthetase, this reached 3.5-fold over control by 4 h. (Fig 23). Contrary to the effect seen with partially purified PDGF, the level of 2'-5' A synthetase returned to background levels within 8 h (not shown). However, this could be explained by the low concentration of PDGF in serum in (< 5 ng/ml).

PDGF alters the level of a 75 kD 2'-5'A binding protein (RNase L) in the cytoplasm of Balb/c-3T3 cells. The level of RNase L was measured by the 2'-5'A binding and crosslinking assays. Cytoplasmic extracts from quiescent Balb/c-3T3 cells treated with PDGF for varying lengths of time were incubated with a labeled derivative of 2'-5' A, (2'-5')pppApApApA[32P]pCp, and UV irradiated. This resulted in the crosslinking of (2'-5')pppApApApA[32P]pCp to RNase L. The crosslinked complex migrates as a single 75 kD protein band in SDS-PAGE. The amount of crosslinked 75 kD protein, RNase L, modestly diminished 30 min after addition of partially purified PDGF (Fig 24). The level increased again transiently 2.5 h after partially purified PDGF addition . The level of detectable crosslinked RNase L was reduced 2-fold by 8 h and 10-fold by 12 h after partially purified PDGF addition, (Fig 24). This low levels persisted for at least 24 h after treatment (Fig 24). Nuclear pellet fractions were obtained from the same cells as the cytoplasmic extracts. These nuclear extracts contained a (2'-5')pppApApApA[32P]pCp-crosslinked protein of 75 kD. Furthermore, the level of this protein also decreased when the cells were exposed to PDGF (not shown).

The reduction in the level of RNase L was reminiscent of the effect seen by Cayley et al. after encephalomyocarditis virus (EMCV)

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Figure 23. Bovine calf serum induces the expression of a 2'-5' A synthetase: Time course of induction. Quiescent Balb/c-3T3 cells were treated with 20% bovine calf serum for varying lengths of time. Cytoplasmic 2'-5' A synthetase was assayed by the polyI:polyC agarose method. The amount of 2'-5' A made is plotted vs. time of exposure of the cells to calf serum.

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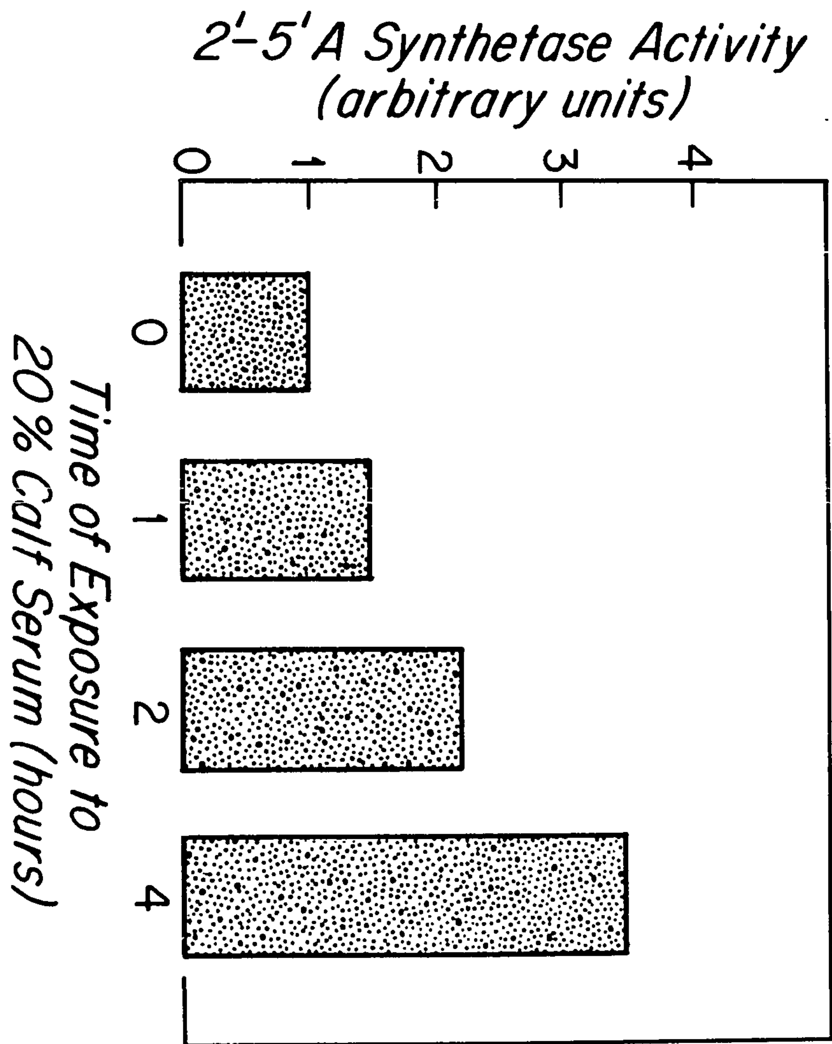

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Figure 24. Partially purified PDGF decreases the level of (2'-5')pppApApApA[32P]pCp binding protein in quiescent Balb/c-3T3 cells. Quiescent Balb/c-3T3 cells were treated with 300 U/ml of partially purified PDGF for varying lengths of time, and cytoplasmic extracts were made as described in the materials and methods section. (2'-5') pppApApApA[32P]pCp was crosslinked to proteins in the cytoplasmic extracts. The crosslinked products were fractionated by SDS-PAGE and visualized by autoradiography. The signal corresponding to the 75 kD (2'-5') pppApApApA[32P]pCp-crosslinked protein was quantified by scanning the autoradiogram. Inset shows duplicate samples for untreated quiescent cells , cells treated with partially purified PDGF for 0.5 h , 2 h , 8 h , 12 h , and 24 h.

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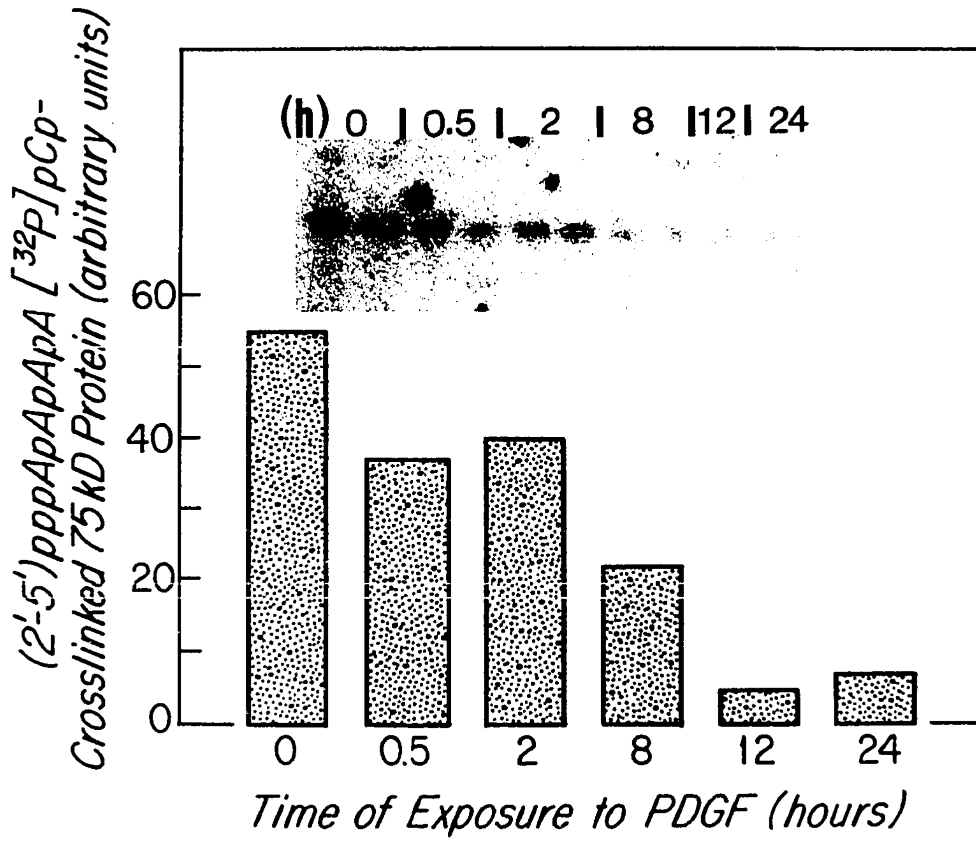

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infection (117). In this case, IFN pretreatment prevented the virally mediated decrease in detectable 2'-5'A binding protein (117). We tested whether or not IFN would abrogate the PDGF induced decrease in detectable RNase L. Balb/c-3T3 cells were treated with 500 U/ml ß-mouse IFN for 18 h prior to the addition of partially purified PDGF. The decrease in the level of RNase L still occured; however, the effect was delayed (not shown).

The 75 kD 2'-5'A binding protein (RNase L) is stable in quiescent Balb/c-3T3 cells. If the 75 kD 2'-5'A binding protein (RNase L) were unstable in quiescent Balb/c-3T3 cells and if partially purified PDGF would inhibit its synthesis the 2'-5'A binding activity would decay rapidly. On the other hand, if the 2'-5'A binding activity were very stable in quiescent cells, the partially purified PDGF mediated decrease would have to involve a labilization of the binding activity. We measured the stability of the 2'-5'A binding activity in cells where protein synthesis was inhibited with cycloheximide. Balb/c-3T3 cells were grown to confluence and made quiescent by growth for 24 h in DMEM supplemented with 2% PPP. These cells were treated with 25 μg/ml cycloheximide for varying lengths of time. Cytoplasmic extracts were crosslinked to (2'-5')pppApApApA[32P]pCp and the crosslinked complex was fractionated in SDS-PAGE and visualized by autoradiography. After 8 h of cycloheximide treatment the level of detectable 75 kD (2'-5')pppApApApA[32P]pCp crosslinked protein only diminished to 87 % of the level in untreated cells (Fig 25). Protein synthesis was inhibited by over 90% as determined by [35S]-methionine incorporation into TCA precipitable products and

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Figure 25. The 75 kD (2'-5')pppApApApA[32P]pCp-crosslinked protein is stable in quiescent Balb/c-3T3 cells. Quiescent Balb/c-3T3 cells were treated with 25 μg/ml (-•-) or 12.5 μg/ml (-o-) of cycloheximide for varying lengths of time. (2'-5') pppApApApA[32P]pCp was crosslinked to proteins in the cytoplasmic extracts. The crosslinked products were fractionated by SDS-PAGE and visualized by autoradiography. The level of the 75 kD protein is plotted against length of time the cells were exposed to cycloheximide at the concentration indicated in the figure. Inset shows autoradiogram with signals corresponding to the 75kD (2'-5')pppApApApA[32P]pCp-crosslinked protein for untreated quiescent cells 0, 8- h, cells treated with 25 μg/ml of cycloheximide for 4 h or 8 h .

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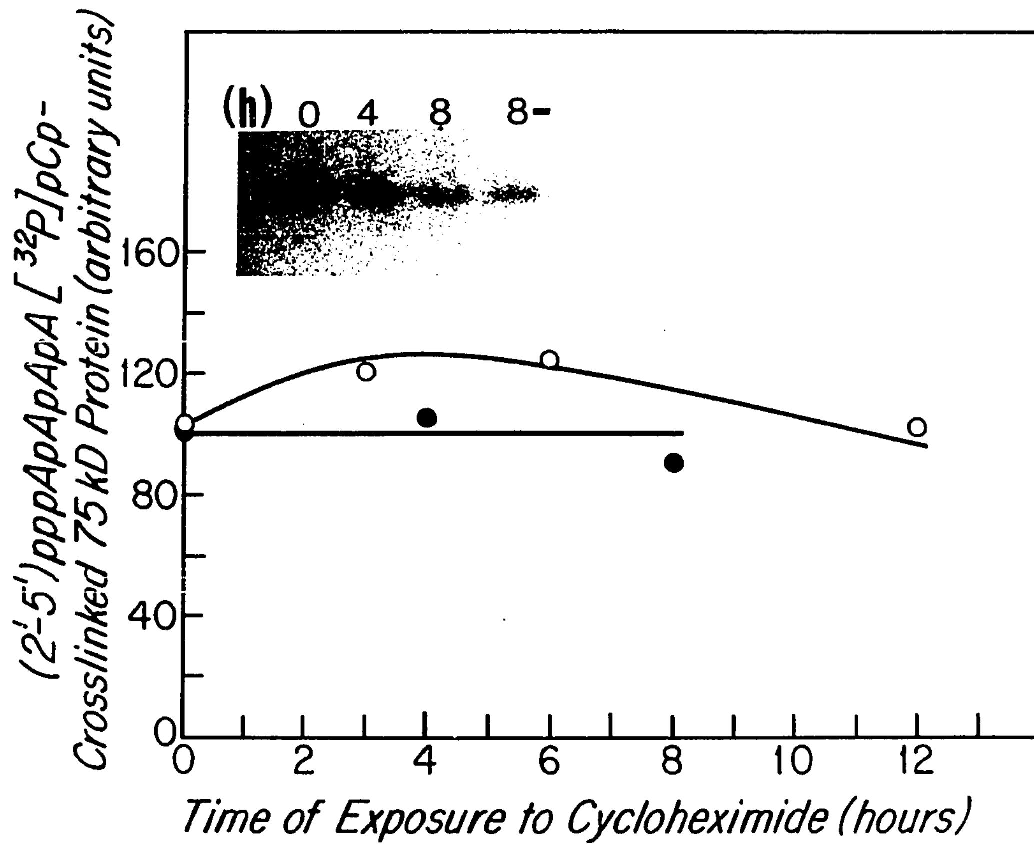

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visualization of labeled proteins by SDS-PAGE and autoradiography (not shown). Cells were not viable beyond 10 h when grown in this concentration of cycloheximide. In order to study longer time points, we treated quiescent Balb/c-3T3 cells with 12.5 µg/ml of cycloheximide (Fig 25). After 12 h of this treatment, the levels of the 75 kD (2'-5')pppApApApA[32P]pCp crosslinked protein did not diminish significantly (Fig 25). The cells remained viable well beyond 12 h in 12.5 µg/ml of cycloheximide, and protein synthesis was inhibited by over 90% (not shown).

IFN inhibits the mitogenic action of PDGF without inhibiting the induction of c-myc RNA . We corroborated the findings of Tominaga and Lengyel (63) and others (13) and showed that mouse ßIFN inhibited the mitogenic action of platelet extract (not shown). Furthermore, we extended these results using purified PDGF (B-chain homodimer) (not shown). In agreement with Tominaga and Lengyel (63) we found that IFN had to be added to the cells for more than 24 h in order to see the mentioned result (not shown).

We tested the effect of ßIFN on the induction of c-myc RNA by PDGF. Quiescent, confluent Balb/c-3T3 cells were treated with 500U/ml of ßIFN for varying lenghts of time and stimulated with 300 PDGF U/ml of platelet extract. Three hours after stimulation total cellular RNA was isolated and analyzed as dsecribed above. c-myc transcripts were detected by hybridization to c-myc cDNA clones containing either exons 1 and 2 or exon 3 sequences and JE transcripts were detected by hybridization with a full length JE cDNA. IFN did not inhibit the PDGF induction of the c-myc or of the

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JE RNAs (not shown). This was the case even when the IFN treatment conditions resulted in a strong inhibition of the mitogenic action of PDGF.

Using a mouse alpha/beta IFN preparation Einat et al obtained a complete inhibtion of the PDGF induction of the c-myc transcript (195). The authors reported that the optimal effect was seen when the cells were exposed simultaneously to the IFN preparation and to low doses of PDGF. In this study Einat et al used a different strain of Balb/c-3T3 from that we used (Adi Kimchi, personal communication). Using the conditions described by these authors we tested whether purified mouse βIFN could also inhibit c-myc RNA accumulation. Quiescent, confluent Balb/c-3T3 cells (see Fig 26, and the figure legend for details) were exposed to either βIFN (100 or 1000 U/ml), or 22 PGDF U/ml of platelet extract or both simultaneously. Two hours later total RNA was isolated and analyzed as described above. 30 U/ml of PDGF induced the accumulation of the c-myc (Fig 26) and JE (not shown) transcripts, whereas βIFN had no such activity. Furthermore, co-addition of βIFN and PDGF did not decrease the level of c-myc (Fig 26) or JE (not shown) transcripts detected, relative to the levels when cells were treated with PDGF alone. On the other hand, with 1000 U/ml of βIFN a slight superinduction of these RNAs was seen, in agreement with a previous observation of Tominaga and Lengyel (63).

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Figure 26. ß-IFN treatment does not inhibit induction of c-myc by PDGF. Balb/c-3T3 cells were grown to confluence. The confluent cells were made quiescent by growth overnight in either DMEM supplemented with 5% PPP or DMEM supplemented with 1% spent calf serum (CS). The quiescent cells were exposed simultaneously to one or more of the following: 22 U/ml PDGF, 100 U/ml of ß-IFN or 1000 U/ml of ß-IFN, as indicated in the figure. Two hours later total RNA was extracted and purified as described above. Levels of c-myc RNA were measured using Northern blot analysis. Hybridization was with a cDNA clone of the murine c-myc spanning exons 1 and 2 of the gene. The 2.2 kb c-myc transcript was visulalized by autoradiography and the levels of the transcript quantified with a soft laser scanner.

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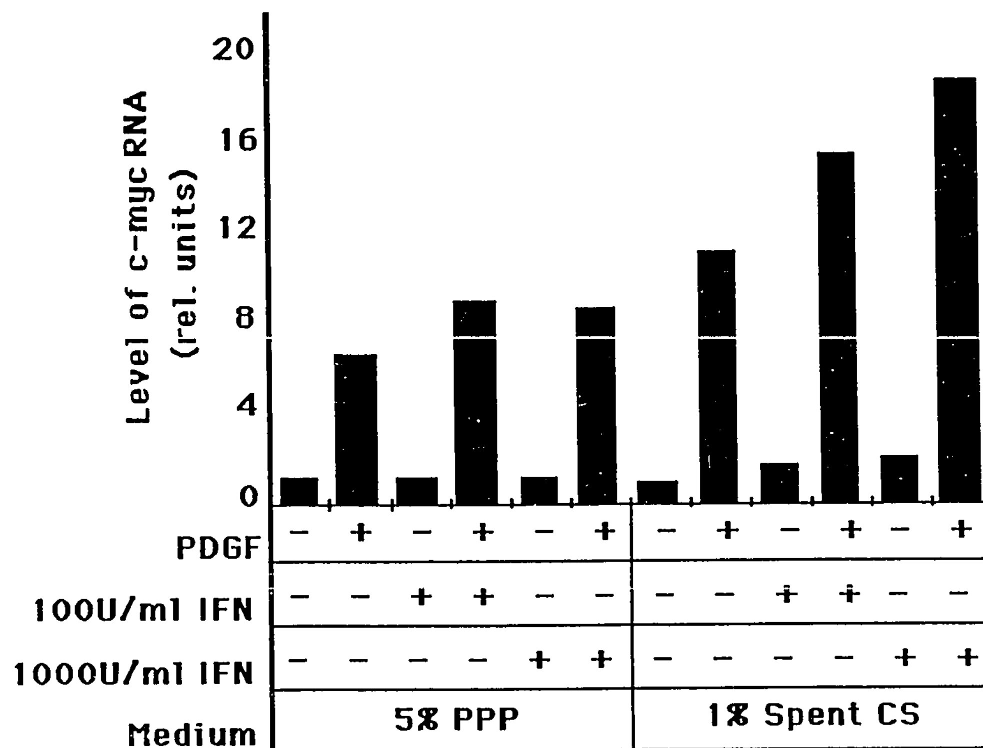

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DISCUSSION

We have been interested in exploring the biochemical interactions between the 2'-5'A system and growth regulatory circuits in cells. In order to study these we used the well characterized Balb/c-3T3 cell line and in particular focused on the transition from quiescence (G0) to growth mediated by PDGF. We evaluated the fluctuations in the levels of the 2'-5' A synthetase and the RNase L during this transition.

PDGF induces the expression of 2'-5' A synthetase transcripts and a 2'-5' A synthetase in Balb/c-3T3 cells . Zullo et al had previously shown that PDGF induces the expression of a 1.8 kb transcript that hybridizes to a human 2'-5' A synthetase oligonucleotide probe (13). Using a murine 2'-5' A synthetase cDNA probe we have confirmed this finding. Moreover, we have characterized this induction further by determining the kinetics of induction (Fig 18), the PDGF dose dependence (not shown) and the relative specificity of this induction by different growth factors (Fig 20). The mechanism of this induction is not completely understood. Work of B.R.G. Williams and coworkers suggests that the human 2'-5' A synthetase promoter region is serum inducible. Therefore, the induction we observe may be mediated , at least in part, by a transcriptional mechanism (M. Rutheford, personal communication).

A very large transcript (3.6-4 kb) that hybridized to the 2'-5' A synthetase cDNA was observed to be inducible by partially purified PDGF (Fig 18, panel B), calf serum (not shown) and ß-IFN

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(Fig 18, panel A). Furthermore, accumulation of this RNA was induced with faster kinetics than the 1.8 kb transcript by both ßIFN and partially purified PDGF (Fig 18). This behavior is compatible with at least two alternatives. First, this RNA could be a precursor of the 1.8 kb RNA. Second, this large transcript could be a distinct 2'-5' A synthetase RNA inducible by PDGF and IFN. St Laurent et al showed that mouse EAT cells contain two IFN inducible 2'-5' A synthetase RNAs,1.5 kb and 3.8 kb, and at least two 2'-5' A synthetases, 20-30 kD and 100 kD (84,84b). Moreover, Mallucci and coworkers have shown that in synchronous mouse embryo fibroblasts, a very large 2'-5' A transcript is induced during the S phase (233).

We show here that partially purified PDGF, calf serum and pure PDGF induced the expression of a 2'-5' A synthetase detectable in the cytoplasmic fraction of Balb/c-3T3 cells (Figs 19,21 & 23). The induction of the 2'-5' A synthetase was detectable as early as 2 h after addition of partially purified PDGF (Fig 19). Moreover, this induction was strong, with maximum values around 10-fold. In one experiment, we tested nuclear fraction extracts of Balb/c-3T3 cells for 2'-5' A synthetase activity. This nuclear activity was induced by PDGF (not shown).

In all the 2'-5' A synthetase assays presented here, the level of enzyme was quantified by the amount of ATP converted to 2'-5' A after a long incubation. The level of 2'-5' A, however, is determined by its rate of synthesis and its rate of degradation. Two facts make us confident that our determinations principally reflect synthesis. First, the polyI:polyC agarose assay detects the

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activity of a parttially purified enzyme, retained on the polyI:polyC agarose beads. This assay is usually not hindered by the presence of a 2'-5' phosphodiesterase. The polyI:polyC agarose assay agreed well with the solution assay. Second, the degradation rates of (2'-5')pppApApApA[32P]pCp in the crude extracts were similar regardless of the growth condition of the cells from which the extracts were obtained. Moreover, the fact that we show here the induction of a 2'-5' A synthetase RNA by PDGF supports our data showing that the enzyme level is elevated.

In both mouse and human cells there are at least four distinct 2'-5' A synthetases, all or some of which can be expressed in the same cells (84,84b). We do not know which 2'-5' A synthetase is responsible for the activity increase induced by PDGF.

Another question still unanswered is whether or not the 2'-5' A synthetases were active in vivo. We assayed for the presence of 2'-5' A in trichloroacetic acid extracts of Balb/c-3T3 cells to investigate whether the 2'-5' A synthetase was active in vivo. Determinations of 2'-5' A levels yielded some conflicting results; however, there was a suggestion of an increase in 2'-5' A within minutes after PDGF stimulation (not shown). Nerve growth factor was shown to elevate the levels of 2'-5' A in PC-12 cells (235). Interestingly, the increase of 2'-5' A in these cells preceded both the induction of a 2'-5' A synthetase and the reduction of 2'-5' phosphodiesterase activity (235). These data lead to a very intriguing question: What is the dsRNA that activates the 2'-5' A synthetase in vivo? The possibility exists that PDGF and NGF induce the expression of endogenous dsRNA , which could activate

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the 2'-5' A synthetase. Moreover, this dsRNA could trans-activate the PDGF inducible genes that respond to exogenous polyI:polyC (13).

Is the induction of the 2'-5'A synthetases by PDGF mediated by an IFN? Several data suggest that this is not the case. First, PDGF induces the level of the 2'-5'A synthetase transcripts in the presence of cycloheximide. Second, the kinetics of induction of PDGF and IFN are similar (Fig 18). This data fit best a direct induction by PDGF. Third, the relative inductions of the 1.8 kb and 4.0 kb transcripts differ for IFN and for PDGF (Fig 18).

Partially purified PDGF diminishes the level of RNase L in Balb/c-3T3 cells. Partially purified PDGF treatment decreased the level of the 75 kD 2'-5'A binding protein. There was an early, moderate and seemingly transient decrease, followed by a late, profound and persistent decrease. The early decrease was only about 30 to 40% and seemed to be partially reversed. A similar effect was seen when Daudi cells were stimulated to grow (B. Lebleu, personal communication). The later effect ranged from 50% to over 95% decrease in the detectable levels of 75 kD 2'-5'A binding protein in quiescent cells. The level reached a minimum by 12 h and persisted for at least 24 h post partially purified PDGF addition.

The mechanism underlying this phenomenon is not understood, but a few posssible explanations deserve note. It is possible that the lower levels of 2'-5'A binding activity detected are the consequence of RNase L binding in vivo to endogenous 2'-5'A or a 2'-5'A-like substance (80) . Once bound the 2'-5'A would exchange with (2'-5')pppApApApA[32P]pCp too slowly to be detected in the binding assays (not shown). The related possibility that free

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endogenous 2'-5' A was present in the cytoplasmic extracts was made highly unlikely by our failure to detect it in mixing experiments (not shown).

Other mechanisms may involve a decrease in the level of RNase L protein. We showed that the half life of the 2'-5'A binding activity in quiescent cells was much longer than 12 h. Therefore, in order to decrease the 2'-5' A binding activity PDGF must labilize it. It is possible that the factors responsible for labilization of the 2'-5' A binding protein are themselves very labile proteins; this would give a false stabilization of 2'-5'A binding protein in cycloheximide treated cells, not reflecting a true half life for the 2'-5' A binding activity.

The effect of PDGF on the cytoplasmic 75 kD 2'-5' A binding protein, RNase L, is intriguing. The only known fuction of 2'-5' A is to bind and activate the RNase L; paradoxically, PDGF induced the 2'-5' A synthetases, whereas it decreases the level of RNase L. However, the effect on the RNase L is delayed relative to that on the 2'-5' A synthetases. This delay could insure that the 2'-5' A system would be induced early after PDGF stimulation. At later times, the effect on the RNase L could negatively regulate the 2'-5' A system.

There are several reports suggesting that growing cells have lower levels of RNase L than quiescent cells. This was shown in NIH 3T3 and JLS-V9R mouse cells, and was implied from data with embryonal carcinoma cell lines and mouse embryos (142,143,145). The decrease of the RNase L levels may be required for the mitogenic effect of PDGF.

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The induction of the 2'-5' A synthetase may be part of an overall protective program induced by PDGF. The physiological scenario when the PDGF response comes under strong selection pressure is during recovery from injury. PDGF released when platelets aggregate during blood clotting, has many physiological functions. Many of these, including the mitogenic stimulus on fibroblasts, are thought to be involved in wound healing and the reinsulation of the organism from foreign pathogens. Thus, it seems advantageous for PDGF to induce the level of a 2'-5'A synthetase. This enzyme could confer to the PDGF stimulated cells a latent anti-viral system.

IFN inhibits the mitogenic action of PDGF without inhibiting the induction of c-myc RNA. It is clear from the data presented above, and from many published reports, that IFNs antagonize strongly the mitogenic effect of PDGF on Balb/c-3T3 cells (13,63,195). Moreover we have shown that IFN decreases the growth rate of sparse Balb/c-3T3 cells (not shown). However, we and others (63, B. Cochran, personal communication) could not detect any inhibition of the PDGF mediated induction of c-myc RNA by IFN. We do not know why our results do not agree with Einat et al, although the only obvious difference is that we used pure ßIFN whereas they used a preparation that contained alpha and beta IFNs. We have preliminary evidence that TGFß may decrease the level of induction of JE when added simultaneously with PDGF (not shown). It is tempting to speculate that a non-IFN activity present in the alpha/beta IFN preparation used by Einat et al is responsible for the effects described by these authors.

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One may argue that the 2'-5' A system is responsible for the very short half life of the c-myc RNA induced by PDGF under these conditions, and thus for the transient expression of the c-myc RNA (Alberta, Garcia-Blanco and Stiles unpublished data). Although the level of 2'-5' A synthetase was low in confluent quiescent cells, there was detectable activity, probably enough to insure an active 2'-5'A system (Fig. 21). Confluent quiescent Balb/c-3T3 cells were pre-treated with ß IFN, inducing a 2'-5' A synthetase, however, the induction of c-myc RNA by PDGF was unaffected (63). This could imply that the 2'-5' A system may not be involved in labilizing c-myc transcripts under these conditions. The situation may be very different in PDGF stimulated subconfluent Balb/c-3T3 cells, data of Dean et al. suggest that the persistent induction of c-myc RNA by serum in these cells is controlled by transcriptional and post-transcriptional mechanisms (241).

The causal relationships between cell growth and the 2'-5' A system remain to be discerned. The findings presented here open several avenues of questions: Is the decrease in the level of RNase L necessary for cell growth? Is the 2'-5' A system controlling the level of RNAs that encode proteins required for the transition from G0 to cell division? Is the 2'-5' A system regulated in quiescent subconfluent cells and is it regulated by other growth factors?

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CHAPTER SEVEN

PDGF INDUCES THE EXPRESSION OF ENDOGENOUS dsRNAS. A HYPOTHESIS.

PDGF and double stranded RNA induce the expression of an overlapping set of genes. The events which occur soon after quiescent Balb/c-3T3 cells are exposed to PDGF must include the formation or alteration of signal transducers. These signals will regulate the mitogenic response. Some of these may be induced by other growth factors in other cells. A series of biochemical events observed after PDGF treatment of Balb/c-3T3 cells have also been described in other cell systems and with other mitogenic stimuli (242). Genes, termed competence genes, are expressed at higher levels after PDGF is added to cells (238). As early as ten minutes after addition of PDGF there is an increase in the transcription of the c-fos gene, and a subequent increase in the c-fos mRNA level; quickly followed by a decline in both (243). Soon thereafter a transient increase in the expression of several other competence genes: c-myc, r-fos, JE, KC and the gene encoding ornithine decarboxylase is observed (13,238,243). The expression of these genes is probably controlled at the transcriptional (243)

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and post-transcriptional levels (241). The latter may be regulated at least in part by the 2'-5'A system.

Stiles and co-workers showed that polyI:poly C, a synthetic double stranded RNA (dsRNA), mimicks many of the effects of PDGF when added to quiescent Balb/c-3T3 cells (13). dsRNA and PDGF induce the expression of c-fos, c-myc, JE, and KC mRNAs (13). The induction of these genes by dsRNA appears to be faster than that by PDGF. Infection with vesicular stomatitis virus (VSV) of quiescent Balb/c-3T3 cells, which most probably results in high levels of intracellular dsRNA, also induces expression of several competence genes (13). It had previously been shown that Newcastle Disease virus induces expression of c-myc in L cells (250).

Addition of polyI:polyC to quiescent Balb/c-3T3 cells is not, by itself, a mitogenic stimulus. Nevertheless, addition together with the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a weak mitogen, results in a mitogenic stimulus equivalent to that seen with PDGF (13). It has been shown that dsRNA is mitogenic for human fibroblasts in culture (244, 249).

Given that polyI:polyC and PDGF were activating a common set of genes, and that polyI:polyC activates the IFN genes, it was logical to ask whether or not PDGF would activate the IFN gene in Balb/c-3T3 cells. It had been known for years that mitogenic stimulation of lymphocytes induces IFN production and induced competence genes (242). Moreover, a previous report showed that IFN was induced when quiescent human fibroblasts are stimulated with EGF (89). PDGF induced the expression of βIFN mRNA in Balb/c-3T3 cells (13); however this result has not been reproducible

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(C. Stiles, personal communication).

PDGF induces the expression of endogenous dsRNAs : A hypothesis. The following is the hypothesis I postulated in order to explain the above data. i) PDGF (and maybe other growth factors) induce the expression of dsRNA, ii) the dsRNA trans-activates dsRNA dependent genes, iii) the dsRNA activates the synthetases, which in turn results in the activation of the RNase L, this could post-transcriptionally regulate the expression of several genes, iv) the dsRNA are encoded by repeated DNA elements. Although we have assumed these RNAs are confined to the cell nucleus it is possible that they exist also in the cytoplasm, possibly activating the dsRNA dependent protein kinase.

The idea that dsRNAs serve as intracellular signals is not new, during viral infection, virally encoded dsRNAs induce the expression of IFN and activate the (2'-5') oligoadenylate synthetase and dsRNA dependent kinase (22). I was humbled to find that what I thought was a completely original hypothesis had in part been proposed by Davidson and Britten in 1979 (282). Furthermore, the potential role of RNA as regulatory molecules was discussed by Dickson and Robertson in 1976 (283).

In a series of recent experiments we obtained data in favor of the above hypothesis. These experiments need to be extensively confirmed and therefore are only mentioned here. We found that PDGF induces the expression of RNAs resistant to digestion by RNases A and T1. This was done by labeling cellular RNA in vivo. The extracted RNAs were digested under conditions

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where dsRNAs are spared, whereas single stranded RNAs are digested. The PDGF inducible, RNase A/T1 resistant RNAs were found to activate a 2'-5' A synthetase. dsRNA is the only known activator of the 2'-5' A synthetase. The products of the 2'-5' A synthetase reactions activated by the RNase A/T1 resistant RNAs were shown to co-migrate with authentic 2'-5'A on TLC and to compete with (2'-5')pppApApApA[32P]pCp for binding to a partially purified RNase L . These RNAs were shown to be sensitive to digestion by dsRNA specific RNases: RNases V1 and III. Digestion with RNase V1 and III resulted in both a loss of detectable radioactive label in RNase A/T1 resistant RNA fraction and a loss of 2'-5' A synthetase activation. Therefore we can conclude that RNAs extracted from cells exposed to PDGF contain higher levels of dsRNA than RNAs extracted from quiescent cells. Hence, PDGF stimulated cells contain higher levels of RNA complementary sequences than quiescent cells. It is reasonable to assume that at least some of these form dsRNA in vivo.

Complementary RNAs exist in cells and there is evidence for the formation of dsRNA in vivo. Measurement of dsRNA using selective intercalating dyes have revealed higher levels of dsRNA in transformed cells than in normal cells (245,246). Furthermore lymphocytes stimulated with a mitogen were shown to have higher levels of these RNAs than quiescent lymphocytes (246). On the other hand, one report in the literature states that growth decreases the levels of dsRNAs measured by differential elution from CF-11 columns (247).

dsRNA has been detected in hnRNA, but not in mRNA. dsRNA

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has been identified by its resistance to A/T1 (252,253), but not to RNase III digestion (252,254), and in some cases has been shown to activate a synthetase activity (252,160). Most of these studies used actively growing transformed cells. Recently transcriptinal units in opposite orientation with overlapping 3' ends were described in drosophila (254b) and mice (254c).

dsRNA has also been detected by in situ immunofluorescence with anti-dsRNA antibodies (255,256). We tried this method with poor results because of problems growing the Balb/c-3T3 cells to quiescence in glass slips .

PDGF inducible dsRNAs may be encoded by repeated DNA elements. RNase A/T1 resistant RNAs from rat (257), mouse (253,258,259) and human (254) cells and tissues were shown to be encoded mostly by repeated DNA elements, both long interspersed elements (LINEs) and short interspersed elements (SINEs). In the mouse the cDNA clones obtained using these RNAs as template were of three size classes: dsRNA-A were identified as sequences homologous to LINEs, dsRNA-B to SINEs (in particular the B1 and B2 elements), and dsRNA-C were very small and heterogenous RNAs (253,258,259,260).

The expression of repeated elements is elevated during early embryogenesis (261-265), in transformed cells (265-272) and, in mitogen stimulated cells (273-278). Furthermore, the *blym* gene, was shown to be a LINE (279). We probed a Northern blot with a labeled DNA segment from a B2 clone and observed a fast induction by PDGF of transcripts of the appropriate size for B2 elements (not shown). In vitro labeled RNase A/T1 resistant RNA, from Balb/c-

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3T3 cells exposed to PDGF, was used as a probe on a northern blot. These hybridized to transcripts with appropriate size for a B2 element RNA. On a genomic southern this RNA probe hybridized to a smear, reminiscent of that seen when genomic southerns are probed with a repeated element (Zullo and Garcia-Blanco, unpublished data).

I propose that the growth regulated increase in the levels of dsRNA is mediated by increased expression of repeated elements. These elements could provide a highly amplified signal. These so called selfish DNA segments (280,281) may have been subverted by the process of natural selection.

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CHAPTER EIGHT
FUTURE DIRECTIONS

Mechanisms by which interferons inhibit the expression of DNA viruses. I showed that IFN treatment inhibits the expression of Tag, SV40 early RNA, and viral replication in SV40 infected cells. Furthermore, IFN treatment selectively inhibits the expression of the viral Tag, but not the cellular (endogenous) Tag, in SV40 transformed cells superinfected with SV40. IFN treatment inhibits the expression of SV40 DNA transfected into cells. Moreover, IFN inhibits the expression of a CAT gene hybrid driven by cellular promoter-enhancer sequences upon transfection into cells. The same CAT hybrid is induced by IFN when stably integrated in cells. The effect on the expression of newly introduced genes may represent a general mechanism of resistance that evolved to prevent invasion by foreign genomes. My assumption is that the inhibition of the expression of newly introduced genes is mediated by mechanisms similar to those involved in the inhibition of SV40 viral infection.

The mechanism by which IFNs inhibit the expression of SV40 early RNA remains a mystery. I return to the three questions

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posed in chapter one: What is(are) the step(s) in the viral life cycle which is(are) primarily inhibited by IFNs? In SV40 infections the answer is not definite. Probable targets for the inhibition, however, have to be localized to the nucleus and most probably occur after viral uncoating and before the accumulation of early pre-mRNAs. In vitro systems need to be established to study whether or not there is a block at or before transcription . Alternatively, the transcripts can be synthezised but are rapidly degraded in IFN treated cells.

What are the cellular factors involved in the inhibition? Unknown. An interesting possibility is that the product of c-myc is involved in the conversion of silent to active genes. IFN could lower the levels of c-myc and therefore inhibit the expression of newly introduced genes. This possibility can be tested in cells that are resistant to the IFN-mediated inhibition of c-myc expression. Adenovirus, which carries in its genome a trans-acting function (E1A) is very insensitive to IFN.

What are the mechanisms of the inhibition? The answer to this question awaits the elucidation of the first two.

Analysis of the inhibition of gene expression may lead to a better understanding of the anti-viral effect of IFNs especially with regard to DNA viruses. DNA viruses are, in general, less sensitive to IFNs than RNA viruses. This may be in part due to very efficient anti-IFN systems that these viruses have evolved. From a different point of view, one may say that these viruses mimick or parasitise cellular processes better, thus making recognition by the IFN system more difficult.

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Many DNA viruses replicate in the nucleus of the cell. Our knowledge of IFN-inducible systems in the nucleus is very primitive compared to that that has accumulated for cytoplasmic IFN-inducible systems. The study of nuclear anti-viral mechanisms is important for a complete understanding of the IFN system.

Studies of the 2'-5'A system: The RNase L. I presented data on 2'-5'A binding proteins in different mammalian extracts from cells and tissues. I also showed the surprising finding that wheat extracts contain a discrete 2'-5'A binding protein. Furthermore, I showed the scheme that we developed to partially purify the RNase L from bovine spleen extracts; and showed data on the parts of this scheme that we have accomplished to date. Our purification scheme provides us with generous quantities of RNase L and a method that separates or inactivates enzymes that degrade 2'-5'A.

The immediate plans after the purification are 1) to sequence the protein, in order to make oligonucleotide probes. These probes will be used to screen bovine cDNA libraries as well as mouse and human libraries.

2) To immunize mice and rabbits with pure enzyme. During the purification we immunized mice, of several strains and using several methods of immunization, but never obtained a producer of antibodies. Antibodies would offer an alternative method of cloning the RNase L by screening of expression libraries. An alternative method for the production of antibodies to the RNase L is to make anti-idiotypic antibodies against antibodies to 2'-5'A. I would target the antibodies that inhibit binding of the 2'-5'A to the RNase

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L. The antibodies obtained, if any, could recognize all 2'-5'A binding proteins.

A direct method for cloning the enzyme in the absence of antibodies or any knowledge of its sequence would take advantage of the specific binding of the 2'-5'A to the enzyme. cDNA expression libraries, such as those cloned in the pUC or lamda gt11 vectors can be used to express eukaryotic proteins in E. coli. A bacterial colony containing a gene encoding a 2'-5'A binding protein could be detected using the solution binding assay described above, or by direct binding to the proteins on filters. In preliminary experiments I showed that lamda gt10 infected E. coli did not have a detectable 2'-5' A binding activity. Furthermore partially purified bovine spleen RNase L still bound 2'-5'A when mixed with these bacterial extracts. A similar method is being used to identify trans-acting factors by screening expression libraries with labeled DNA fragments containing the cis-acting elements bound by these trans-acting factors (P. Sharp, personal communication).

PDGF and the 2'-5'A system. Exposure of PDGF increases the levels of a (2'-5')oligoadenylate synthetase and reduces the detectable levels of a cytoplasmic (RNase L) and a nuclear 75 kD 2'-5'A binding protein. Furthermore, I presented data showing that PDGF induces the expression of two (2'-5')oligoadenylate synthetase transcripts. This work was done in close collaboration with members of the laboratory of Dr. Charles Stiles.

IFN interferes with the mitogenic action of PDGF, therefore it is possible to say that IFN induces the formation of

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anti-mitogenic state in cells. This is a state in which cells have a diminished response to mitogens. This provides a parallel model with the anti-viral state, in which cells are poor hosts for viruses. I have shown evidence that IFN does not act by inhibiting the expression of competence gene transcripts. The anti-mitogenic state and the anti-viral state (in particular that for DNA viruses) probably share biochemical circuits.

dsRNAs as gene activators and regulators of the 2'-5'A system. I mentioned preliminary evidience that PDGF induced the expression of complementary RNAs. It is reasonable to assume that these could form double stranded RNA in vivo. These RNAs can trans-activate dsRNA-responsive genes (ß-IFN, c-myc) and also activate the 2'-5'A system, thereby labilizing some mRNAs. It is probable that these dsRNAs are encoded by reiterated DNA elements in the mammalian genome.

The dsRNA present in RNA from PDGF treated cells should be cloned, probably by using RNAs resistant to cleavage by A/T1 present in higher level in PDGF treated cells. This will help to unravel what may be a novel mechanism of trans-activation of genes mediated by RNA or RNA-containing factors.

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