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INDUCTION OF INTERFERON MESSENGER RNA AND EXPRESSION OF CELLULAR ONCOGENES IN HUMAN LYMPHOBLASTOID CELLS

DISSERTATION

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Namalva is the name given to a unique human B lymphoblastoid cell line that originated from a Burkitt lymphoma and which produces a large amount of alpha and a small amount of beta interferon (IFN) following induction by Sendai virus. The induction of IFN-α messenger RNA (mRNA) in Namalva cells was studied at the transcriptional level. The amount of IFN-α mRNA reached its maximum six hours after induction and then slowly declined. The interferon titer did not reach its maximum value until 18 to 24 hours after induction.

Northern hybridization of induced Namalva cell poly(A)+ mRNA with an interferon probe revealed an IFN-α message of approximately 1,200 nucleotide bases (1.2 kb). The integrity of Namalva cell RNA was checked periodically by electrophoresis using a variety of gel systems and/or by Northern hybridization with an oligomeric probe complementary to 18S ribosomal RNA.

It was of interest to know if two cellular oncogenes, namely c-*myc* and c-Ha-*ras*, expressed in several other lymphoblastoid cell lines, would be expressed in Namalva

cells. Daudi lymphoblastoid cells and normal human leukocytes served as the positive and the negative controls, respectively.

Hybridization of a c-myc probe to Namalva cell RNA revealed a characteristic 2.7 kb transcript and two transcripts larger than 23 kb. Northern hybridization, using a c-Ha-ras probe, revealed two transcripts with approximate sizes of 1.8 and 6.1 kb. These data indicate that the genes c-myc and c-Ha-ras were expressed in Namalva cells.

The amount of c-myc message detected in Namalva cell RNA was about one-tenth that of Daudi cell RNA, whereas no difference in the amount of the c-Ha-ras message was observed between the two cell lines.

The results of c-myc and c-Ha-ras hybridization indicate that the ratio of c-myc to c-Ha-ras expression is much lower in Namalva cells than in Daudi cells.

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CHAPTER I

INTRODUCTION

History

In 1930, Theiler (114) presented a new laboratory model for studies on the action of yellow fever virus on mice. He showed that mice were susceptible to the virus when injected intracerebrally and that the results were essentially the same when the virus was injected into other parts of the nervous system such as the brain, spinal cord, or the eye. However, when the virus was injected intramuscularly, intraperitoneally, or intradermally, it rarely resulted in infection and death. In these mice, immunity to subsequent intracerebral injection of virus was often noted.

Hoskins (52) noted that following inoculation of neurotropic and viscerotropic[1] yellow fever viruses in *Macacus rhesus* monkeys simultaneously, and at intervals of 16, 19, 21, 24, 30, and 48 hours a definite protection against the latter resulted. Out of 15 monkeys

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[1]The term "neurotropic virus" is used to designate yellow fever virus of the "French Stain" which was isolated by Sellards in Dakar and which has been carried through the central nervous tissue cells of white mice for more than 200 passages. The term "viscerotropic virus" is used to designate the strain of yellow fever virus which has been carried in *Macacus rhesus* ever since its isolation from a human patient in 1927 and which produces visceral lesions in and is highly fatal to *M. rhesus*.

receiving these viruses simultaneously, 13 survived while two died of the infection. Administration of the neurotropic virus from 16 to 24 hours after the viscerotropic virus resulted in survival of only 12 of 21 monkeys. No protective effect was observed when the neurotropic virus was injected after an interval of 48 hours. The experiment revealed that when intervals of injection were not more than 20 hours, the neurotropic virus gave ample protection against viscerotropic virus. The results are not surprising when one considers the short incubation period of the disease in the Rhesus monkey. Hoskins offered no explanation for this protective effect since his attempts to find a protective substance in the brains of mice dying from encephalitis due to the yellow fever virus were fruitless.

Several other investigators have reported the same type of phenomenon with plant viruses. McKinney (83) inoculated tobacco plants with common mosaic virus and, when symptoms appeared, inoculated the plants with yellow mosaic virus. The inoculation with common mosaic virus was found to protect against development of yellow mosaic disease.

Salaman (92) worked with two strains of the X virus of potatoes. One strain caused severe disease and was designated type L while the other caused a mild form of the disease and was designated type G. He showed that the inoculation of tobacco and Jimson weed plants with the type

G protected them against disease upon inoculation with the type L virus.

Findlay and MacCallum (37), being aware of the protective phenomenon in plants, tried in 1939 to confirm and clarify Hoskins' study (52). They inoculated eleven monkeys with the pantropic strain of Rift Valley fever virus and two hours later with the pantropic strain of yellow fever virus. Seven out of eleven monkeys were protected from the fatal effects of yellow fever virus. Upon inoculation of mice, intraperitoneally, with mixtures of neurotropic yellow fever virus and pantropic Rift Valley fever virus, they noticed a delay in the death of mice and even complete protection in a few cases. Their experiments firmly established the now well-known phenomenon of "viral interference" (37). They concluded that active virus particles protected certain cells from attack by other viruses.

In 1940, Jungeblut and Sanders (63) immunized monkeys with live, murine strain poliomyelitis virus. They found that these monkeys resisted infection on subsequent inoculation with the homologous poliomyelitis monkey virus. Apparently, the murine virus interfered with the multiplication of poliomyelitis monkey virus. They believed this phenomenon to be analogous to that described by Findlay and MacCallum (37).

Three years later, Henle and Henle (47) partially inactivated viable influenza virus by means of heat or ultraviolet radiation. Embryonated eggs were injected with the active virus and also with virus partially inactivated by heat or by ultraviolet radiation. One half of the embryonated eggs were injected with the active virus and with partially inactivated virus concurrently while the other half was injected initially with the active virus and three hours later with the partially inactivated virus. In both cases, enumeration of viable virus particles in allantoic fluid indicated a decrease in the multiplication of active virus. Apparently the inactivated virus had interfered with the propagation of active influenza virus.

Ziegler et al. (131) confirmed the work of Henle and Henle (47). They found that chick embyros inoculated with influenza A or B viruses failed to support multiplication of either virus upon challenge.

In 1946, Lennette and Koprowski (72) demonstrated viral interference in mouse brain tissues in vitro. They found growth of the Asibi strain of yellow fever virus and the heterologous West Nile virus in tissue cultures to be suppressed by the 17DD high yield strain of yellow fever virus. The authors urged the use of tissue culture techniques for the further study of the mechanism of viral interference.

Allantoic injection of the chick embryo with NWS (Stuart-Harris' "neuro-flu" strain influenza A virus), generally does not produce hemorrhagic brain lesions, however, if the virus is introduced by intravenous inoculation, a fatal hemorrhagic infection results. Burnet and Fraser (19) injected chick embryos in the allantoic cavity with MEL (Melbourne), a non-invasive strain of influenza A virus, followed by strain NWS intravenously 18 to 20 hours later. They observed no hemorrhagic lesions in the embryo. Apparently the primary infection by MEL had protected the embryos from the development of hemorrhagic lesions upon intravenous inoculation of NWS.

Several investigators have studied inhibitors of viral interference. Depoux and Isaacs (31) described the effect of the receptor-destroying enzyme, RDE, of *Vibrio cholerae* on interference between influenza and vaccinia viruses on the chick chorion. The administration of RDE together with, or two hours after, chorionic inoculation of influenza virus lowered the ability of the influenza virus to interfere with growth of vaccinia and the production of a soluble vaccinia antigen.

2,5-Dimethylbenzimidazole, a reversible inhibitor of influenza B virus (109), was found to inhibit viral interference caused by inactivated influenza A (WS strain) or influenza B (Lee strain) viruses (118).

These various early investigations failed to elucidate the real nature of viral interference. A new era in the study of viral interference began in 1957 when Lindenmann and Isaacs (56, 57), working at the National Institute for Medical Research in London, England, added heat-inactivated influenza virus (MEL strain of influenza A) to pieces of chicken egg chorioallantoic membrane. They washed the membranes to remove unabsorbed virus and then incubated them in the 37oC incubator for a few hours. Thereafter, these membranes were removed from the fluid and replaced with fresh tissues. After incubating the fresh membranes for 24 hours, live MEL virus was added and the tissues were further incubated. Replication of the influenza virus was found to be inhibited in the new tissues. Thus, after being exposed to the heat-inactivated influenza virus, the cells had produced a substance that conferred interference on the fresh pieces of chorioallantoic membrane. This substance was named "interferon" by Isaacs and Lindenmann (56, 57, 105). The same phenomenon was also reported by Cantell and Paucker (20) who found that the exposure of L cells to UV-irradiated Newcastle disease virus resulted in the production of a substance that protected cells in tissue culture upon subsequent challenge by vesicular stomatitis virus.

<u>Definition of Interferon</u>. Many investigators (60, 106, 123, 124) have defined and characterized interferon. Isaacs

(60) referred to it as "an antiviral substance, protein or polypeptide in nature, produced by the cells of many vertebrates in response to virus infection which is antigenically distinct from virus, and acts by conferring cell resistance to the multiplication of a number of different viruses." The current and updated definition of interferon is that given by the Committee on Interferon Nomenclature, which met at the National Institutes of Health in Bethesda, Maryland, on March 7, 1980. According to this committee, interferon is defined as follows.

> To qualify as an interferon, a factor must be a protein which exerts virus non-specific, antiviral activity at least in homologous cells through cellular metabolic processes involving synthesis of both RNA and protein (106).

Physico-chemical Properties of Interferon. In some interferons, the protein is associated with carbohydrates but these probably play no role in antiviral activity. The fact that interferon can be destroyed by proteolytic enzymes but not by nucleotideases, either RNase or DNase, nor by lipases, carbohydrases, or receptor-destroying enzymes, establishes its protein nature (36, 105). Other investigators (57, 59) have shown interferon be a protein free of nucleic acid, and one which can be filtered through a collodion membrane with an average pore diameter of 0.048 um, and which is stable at 2°C for several weeks. It can be inactivated by ether and by a mixture of one part amyl alcohol and two parts chloroform; also it was not

sedimented by centrifugation at 100,000 x g for one-half hour or at 20,000 x g for two hours. Another characteristic of interferon is its stability during prolonged exposure to acid at a pH of 2 (124). Hilleman (48) reported that purified chick embryo interferon was stable for a period of one hour at any pH in the range of 2 to 10 at 25°C and at pH 1 at 4°C. Based on the rate of diffusion through hydrated agar, Porterfield _et_ _al_. (89) estimated that the molecular weight of interferon was less than 80,000 daltons. Using ultracentrifugation analysis, Burk (13) reported the molecular weight of pure chick interferon to be 63,000 daltons. Various molecular weights for interferon have been reported, and these appear to depend on the species of producer cells and type of inducer. For example, after inoculation of mice with Newcastle disease virus, the interferons which subsequently appeared in the sera were found to have molecular weights between 23,500 and 38,000 daltons. On the other hand, the molecular weight of interferon in a chick embryo cell culture induced by _Herpes simplex_ virus was found to be 36,000 daltons while in a human leukocyte cell culture with Sendai virus as inducer, the resulting interferon was found to have a molecular weight of 25,000 daltons (77).

Other Properties of Interferon. All interferon proteins do not exhibit the same degree of antigenicity. In fact, several attempts to produce neutralizing antibody

against interferon have been unsuccessful (14, 75). The difficulty in obtaining interferon antibodies may be due to poor antigenicity of interferon (60), to the unavailability of a sensitive assay system to detect antibody (120), or to difficulty in obtaining sufficient quantities of interferon for injection (78). In 1965, Paucker (88) developed a sensitive test for the titration of antibodies against interferon. This test, which was based on the color change of phenol red indicator in the medium, could detect higher antibody titers than previous methods. Levy-Koening *et al*. (73) were able to obtain antisera to interferon produced by human leukocytes. They did a neutralization assay of four types of human interferon obtained from different sources: peripheral leukocytes (PL), human foreskin cells (HFS), and two amnion cell lines (U and AF-57) against anti-PL interferon serum. They found that the homologous system (for example, PL interferon against anti-PL interferon serum) resulted in the highest neutralizing activity observed.

Another important characteristic of interferon is a degree of species specificity. The activity of duck or chick interferon, for example, is limited to the cells of ducks or chickens (121, 122). Species specificity of interferon has also been shown by Baron *et al*. (3) using mouse and chicken interferon; they found antiviral activity in the homologous system but none in the heterologous system. On the other

hand, some interferons are active on cells from unrelated animals. For example, human leukocyte interferon is active on monkey and bovine cells. Therefore, it has been suggested that the term "defined host-ranges" replace the usual term "species specificity" in the case of interferon (105).

Interferon can inhibit the growth of viruses as well as some other types of microorganisms. Normally, addition of virus to cell cultures results in some form of damage to the cells. However, when cells are incubated with interferon before addition of virus, cell damage can be prevented or at least postponed. The resistance of cells to specific viruses depends on the concentration of interferon used and on the incubation temperature of the cell culture. The kind of inhibitory response to interferon also varies according to the origin of the virus. For example, arthropod-borne viruses (arboviruses) are most sensitive to interferon, whereas Mengo viruses (Picornavirus) are less sensitive (77).

Growth of some chlamydiae, the protozoan <u>Toxoplasma gondii</u>, and bacteria such as <u>Shigella</u> <u>flexneri</u> are also inhibited by interferon-treated cell cultures (78, 105).

Interferon can either prime or block the production of interferon. The addition of low concentrations of interferon to cells before viral induction will enhance its production, a process which is called "priming". Addition of high

concentrations of interferon, on the contrary, generally results in a decrease in interferon production. Therefore, depending on the concentration of interferon and on the length of exposure of the cells to interferon, the production of interferon may be increased (primed) or decreased (blocked) (58, 105). In 1958, Isaacs and Burk (58) treated chick chorioallantoic membranes with interferon and induced the cells with living influenza virus. They noticed high interferon production in primed cells compared with the controls which were not pretreated (primed) with interferon. Vilcek and Rada (119) were the first to report on the blocking phenomenon (105, 119). They treated chick embryo cells with interferon and challenged the cells with tick-borne encephalitis virus. They noticed that even doses of interferon which did not inhibit multiplication of tick-borne encephalitis virus, reduced the production of interferon significantly. Another example of blocking was reported by Lockart (76) who showed that the pretreatment of Western equine encephalitis-induced L cells with high concentrations of interferon inhibited interferon production. In addition to priming and blocking, interferon also has the ability to do the following.

a. <u>Inhibition of cell multiplication</u>. This is a process in which cell propagation is inhibited. Tan (110) assayed cell growth inhibitory and antiviral effects of human interferon on primary human skin fibroblasts

containing different copies of chromosome 21. He assayed the amount of interferon required to inhibit both the *in vitro* growth of cells and the synthesis of vesicular stomatitis virus by a factor of 50 percent of that observed in control cells. Tan concluded that human chromosome 21 has the gene(s) that control the inhibition of growth as well as the antiviral activity (interferon).

b. *Toxicity enhancement*. Induced cells which have been pretreated with interferon exhibit enhanced cytotoxicity. In a study by Joklik and Merigan (61), L cells pretreated with interferon and infected with vaccinia virus began to lyse after six hours of incubation; infected cells not pretreated with interferon did not lyse (61, 105). Toxicity enhancement has also been observed in cells pretreated with interferon and induced with double-stranded RNA (104, 105). When human skin fibroblasts were exposed to double-stranded RNA (poly rI poly rC), the cells remained healthy, whereas interferon-treated cells autolysed after 3 to 6 hours of exposure to the same double-stranded RNA.

c. *Enhancement of function*. An example of this phenomenon is shown in the study of Imanishi *et al*. (55). They showed that the addition of human leukocyte interferon to human peripheral monocytes in culture, enhanced the degree of phagocytosis and increased the number of phagocytic cells produced by the reticuloendothelial system.

d. Inhibition of delayed-type hypersensitivity (DTH). It has been shown that induced swelling of the ears of mice by the topical application of picryl chloride and the swelling of mouse foot pads due to injection of sheep erythrocytes were inhibited by exogenous interferon (79). The interferon used was prepared in mouse L cells or in cells of the L-243 murine cell line induced by Newcastle disease virus.

e. Alteration of cell surface. Another important characteristic of interferon is its ability to alter the surface of the host cell. Mouse L cells treated with L cell interferon showed an increase in electrophoretic mobility as a result of increased cell surface negative charge (69). Another evidence of cell surface alteration is the increase in binding capacity of concanavalin A to Murine Leukemia L1210 cells following treatment with interferon (53); cell surface changes were confirmed by electron microscopy.

Clinical Use of Interferons

The clinical use of interferon has been widely investigated. This subject has been well reviewed by Oldham and Smalley (87), Stewart (105) and Strander (108). Interferons have been used clinically since the early 1960's (105) in the treatment of localized viral infections, herpetic keratitis, and other viral infections. Approximately 1 X 10^5 units of human leukocyte interferon

(in the form of eye drops) administered four times per day to patients with herpetic keratitis produced marked recovery. The keratitis did not recur the following year as expected in comparison to other agents. Interferons have also been used for the treatment of systemic viral infections. For example, patients with Herpes zoster infections were treated with 1 to 3 X 10^6 units of human leukocyte interferon administered intramuscularly three times per week; these patients recovered from the infection within ten days. Interferons have also been used for treatment of several types of cancer including Hodgkin's disease. Daily intramuscular injections of 5 X 10^6 units of human leukocyte interferon for one and one-half months led to remission of tumors in patients with Hodgkin's disease (105).

Production and Purification of Interferon

Several steps are involved in the production of interferon. The first step is priming of the cells to be used for production although this step is considered by many to be optional (66, 99, 101). The next step is induction. There are two classes of inducers, viz., viral and non-viral. Under proper conditions, almost every major virus group, including arboviruses, myxo- and paramyxo-viruses, rhabdoviruses, lymphocytic choriomeningitis virus and diplornaviruses are capable of inducing interferon (51, 105). Depending on the cell system and virus used, there

are "poor" and "good" inducers. Some virus types which are good inducers in one system might be poor inducers in another system. Examples of some non-viral inducers include; (a) bacteria such as *Bordetella pertussis* and *Escherichia coli*, (b) bacterial products such as the endotoxin of *Brucella abortus*, (c) rickettsiae such as *Rickettsia mooseri*, (d) chlamydiae of the trachoma-inclusion conjunctivitis group, (e) protozoa like *Toxoplasma gondii*, (f) mycoplasmas such as *Mycoplasma arthritidis*, (g) fungi such as *Penicillium funiculosum*. (h) mitogens such as phytohemagglutinin (an extract of the red kidney bean, *Phaseolus vulgaris*), and (i) nucleic acids such as double-stranded RNA (poly rI poly rC). It has been shown that the active component of helenine, an interferon inducer, is a double-stranded RNA. In addition to this, synthetic anion polymers such as polycarboxylates and polyphosphates (e.g. dextran phosphate) have also been shown to induce interferon production (41, 105, 120).

In producing and purifying interferon, the amount produced or lost needs to be known precisely in order to calculate the specific activity and dosage for clinical use. To date, several methods of interferon assay have been designed and employed (32, 50, 105, 122). One of the most common is the plaque-reduction assay first introduced by Wagner (122) in 1961. This assay is based on the inhibition of plaque formation in cell monolayers by challenge virus

added to the interferon-treated cells. In this method, the greater the quantity of interferon added to cell monolayers, the greater the reduction in infectious viral particles as reflected in the number of plaque-forming units (105, 122). Another common interferon assay is the cytopathic effect (CPE) assay. In this method, first described by Ho and Enders (50) in 1959, interferon is added to tissue culture cells and after incubation, cytopathic challenge virus is added. The more the interferon in the sample, the lower the cytopathic effect which can be seen on microscopic examination. In any interferon assay system, the interferon titer (units) is the reciprocal of the highest dilution of interferon that is able to inhibit virus growth to a certain degree in a particular assay system. In the plaque reduction assay, for example, the titer is the reciprocal of the highest interferon dilution that inhibits plaque formation to a level of 50 percent when compared to a control system. In the CPE assay, the titer is the highest dilution of interferon that gives 50 percent protection when the virus control is taken as the 100 percent CPE level. Considering the variability in virus infectivity and cell resistance, it is obvious that the different assay systems will give different values for interferon quantity. In addition to this, since the plaque assay system and the CPE activity are not necessarily based on the same reactive components, the assay of interferon will differ with the assay method

employed. Therefore, according to agreement reached by the International Association of Microbiological Societies in 1969, several international interferon standards such as the NIH reference standard have been selected (38, 105, 120). Some of the international interferon standards include human leukocyte interferon prepared from Sendai virus-induced leukocytes; rabbit interferon, obtained from albino rabbits after injection with Newcastle disease virus; and mouse interferon, obtained from Swiss mice after injection with Newcastle disease virus (38). Following induction of cells with interferon inducers, interferon is produced. A mechanism of interferon production has been hypothesized by Burk and others (16, 17, 18, 105). According to this theory, after invasion (induction step, with either infective or inactivated virus) the virus probably uncoats and releases virus component(s) that stimulate the formation of interferon. These viral products probably interact with the host genome directly or indirectly, leading to the synthesis of interferon mRNA and hence to the production of interferon and other related proteins. To harvest interferon, the cells are removed by centrifugation. The supernatant which contains interferon is further processed for purification of the interferon present.

The most commonly employed methods for purifying interferon are given below:

1. Precipitation method. This method, one of the first employed for purifying interferon, takes advantage of the fact that interferon is a protein, and as such can be precipitated by ammonium sulfate (74). Other chemicals such as picric acid and trichloroacetic acid have also been used for this purpose (35, 36). In another method developed later, crude interferon was precipitated by potassium thiocyanate and recovered by further precipitation with ethanol. After several subsequent changes of acidity at pHs ranging from 3.0 to 7.4, partially purified interferon was obtained (21).

2. Ion-exchange chromatography. The use of ion-exchange resins for purifying interferon was first reported by Burk (13, 15). In his study, ammonium sulfate precipitation of interferon was followed by ion-exchange chromatography, first on sulfomethylcellulose at pH 2.0 and then several passes through diethylaminoethylcellulose at pHs ranging from 4.5 to 6.6.

3. Gel filtration. Resins such as Sephadex have been employed in the purification of interferon since it is possible to separate specific protein moieties by passing mixtures of proteins through beds of hydrated resin beads (35).

4. Affinity chromatography. Antibody affinity columns prepared with antiserum against human leukocyte interferon have been used to purify human fibroblast, Namalva and human

leukocyte interferon. By using a column made by coupling highly absorbed anti-leukocyte interferon immunoglobulins to sepharose 4B, this method was found to yield from 2 to 20 X 10^7 units of interferon per milligram of protein in a single-step procedure (5).

In a very recent study (8), a murine monoclonal antibody was described which recognizes porcine leukocyte interferon. It has been suggested that this antibody has affinity for a common epitope of the conserved region in the human alpha interferon system. An antibody affinity column using this antibody has made possible the purification of all species of human alpha interferon from crude material in a single step (8). The availability of a monoclonal antibody also ensures a more consistent preparation of antibody and therefore greater consistency in the purification procedure since the variations associated with the production of antibodies in animal systems are eliminated.

Classification of Interferon

The old classification (106) of interferon was based on the source of interferon. For example, interferon obtained from leukocytes was named type 1, Le (from leukocyte); interferon from fibroblast cells, type 1, F (from fibroblast); interferon from T cells, immune interferon, IIF, or type II; and interferon from B lymphocyte derived cell lines, lymphoblastoid interferon. Today, however,

interferon is classified on the basis of antigenic specificities. According to the new classification, there are interferons designated alpha (IFN-α), beta (IFN-β) and gamma (IFN-γ), instead of leukocyte, fibroblast and immune interferon, respectively. Some interferon preparations, like lymphoblastoid interferon, contain a mixture of alpha and beta (106). One reason for the new classification is that some interferons prepared from different sources are of the same type. For example, alpha-interferon is produced by fibroblasts, lymphoblastoid cells and leukocytes and beta-interferon is produced by leukocytes, lymphoblastoid cells and fibroblasts.

Namalva Lymphoblastoid Cells

Namalva cells represent a B lymphoblastoid cell line derived from the tissues obtained from a young Ugandan girl ill with Burkitt's lymphoma. A biopsy was taken from the patient in Nairobi and the cells were sent to Dr. George Klein in Stockholm, Sweden where they were established as a cell line (39, 86). Namalva cells, like other human lymphoblastoid-derived cells, carry the genome of the Epstein-Barr virus, a characteristic which is apparently needed for their continuous growth (67). The Namalva cell line spontaneously produces small amounts of interferon in the absence of an inducer (1).

Twenty one different lymphoblastoid lines were tested in the first study concerning interferon production from Namalva cells (104). After induction of the 21 lines with 600 hemagglutination units of Sendai virus, the interferon liberated into the medium was assayed. The Namalva cell line was found to be the best producer of alpha-interferon. After a few modifications in the induction system, the titration of Namalva interferon reached 11,000 units per ml (107). To date, more than 170 human lymphoblastoid cell lines have been tested for interferon production and the Namalva line is still considered the best lymphoblastoid interferon producer (22, 24). Namalva cell interferon is similar to human leukocyte interferon (107, 132) in that it is a mixture of about 80 to 90 percent alpha-interferon and 10 to 20 percent beta-interferon (7, 33). The similarity in activity which exists between leukocyte and Namalva interferons has caused several investigators, companies, and research centers around the world to substitute leukocyte interferon with Namalva cell interferon for clinical trials. Following recognition of the Namalva cell line, several attempts have been made to produce interferon from Namalva cells in the massive quantities necessary for clinical use. The range of vessels used for mass production has varied from a 50-liter fermentor (66) to an 800-liter tank (12), and even to a 1,000-liter stainless steel fermentor (39, 40).

Namalva is a transformed cell line which carries the genome of Epstein-Barr virus (67) and many investigators question the use of Namalva interferon in cancer therapy (39). They raise the question of whether the interferon recovered from a transformed line which carries a viral genome is suitable for cancer therapy but the question remains unanswered. Therefore, in places where the use of interferon produced by Namalva cells is permitted for clinical trials, such as the United Kingdom, precautionary measures such as the addition of bacteria and viruses to the crude interferon and their elimination in the purification procedure are carefully monitored to make certain that any viruses produced by the Namalva cell would not be included in the interferon product. This can be done by fairly easy techniques due to the basic differences between interferon and other cell products such as viruses (39).

In Namalva cell interferon research, two major lines of work are being followed at this time. One concerns the production and purification of interferon in large quantities and the other involves studying the molecular events which occur after induction of Namalva cells to produce interferon. The latter can be studied at the transcriptional or the translational level by looking at the level of interferon mRNA synthesis and of interferon production. In this sense, the study of interferon mRNA

synthesis will help to elucidate the chain of transcriptional events that lead to the formation of interferon.

Several researchers have characterized the synthesis of Namalva cell interferon mRNA at the translational level using the oocyte assay technique (9, 26). In this procedure, the mRNAs are isolated after induction of Namalva cells for interferon production. The mRNAs are injected into individual *Xenopus laevis* oocytes by use of a micromanipulator. After an incubation period of 18 hours, the oocytes are homogenized and the supernatants are prepared for interferon assay (102). The interferon produced in the oocytes is the result of translation of interferon mRNA. The level of interferon mRNA synthesis has also been studied using S1 mapping (49). The S1 is an endonuclease enzyme which degrades single-stranded RNA or DNA into 5'-mononucleotides. In this technique, plasmids containing chromosomal interferon genes were cleaved at a specific restriction site, 32P end-labeled and cleaved again with a second appropriate restriction enzyme. In the reported experiment (49), most of the probes were labeled at the 5'- terminus at a restriction cleavage site unique to each gene used as a probe. Therefore, the 32P-labeled end of a mismatched probe was accessible to S1 nuclease digestion. In this way, specific probes were prepared to distinguish between specific interferon genes. The level of interferon mRNA was measured by calculating the number of

interferon mRNA strands per induced Namalva cell.

Oncogenes. To date, several lymphoblastoid cell lines have been shown to express a variety of oncogenes (28, 34, 98, 112). There are several ways that a proto-oncogene can be activated. One mechanism involves overexpression of the proto-oncogene as seen in chicken lymphoma. The DNA of avian leukosis virus integrates proximally to the c-*myc* gene. In this case, increased transcription from the viral promoter is responsible for overexpression of c-*myc* (10, 68). Gene amplification also leads to proto-oncogene activation. Transcription from multiple copies of the gene results in increased expression of the proto-oncogene (70). Other mechanisms include point mutation, activation by retroviruses, and chromosomal translocation or rearrangement (127).

One of the unique features of several Burkitt lymphoma cell lines studied to date concerns c-*myc* expression. The c-*myc* proto-oncogene becomes translocated from its original location on chromosome eight to the end of another chromosome. As a result, the c-*myc* gene is placed in the proximity of immunoglobulin genes which are active in B lymphocytes (54, 127). These translocations occur between chromosomes eight and fourteen, which contain immunoglobulin heavy chain genes, or between eight and two, which contain immunoglobulin *kappa* chain genes, or eight and twenty-two,

which contain immunoglobulin lambda chain genes (10, 27, 29).

The c-myc gene is a retrovirus-associated oncogene first isolated from the genome of avian MC29 myelocytomatosis virus of the chicken. It is located on human chromosome number 8 and on mouse chromosome number 15 (29, 70). The c-myc product (oncogenic protein) is localized in the nucleus (nuclear matrix) and it functions by binding to DNA (54, 70).

The other oncogene studied in this report, c-Ha-ras, is a member of the ras family. To date, three classes of mammalian ras genes have been reported (11). Two classes of ras genes, namely Ha-ras and Ki-ras, are retrovirus-associated oncogenes (70). They have been isolated from the genome of Harvey murine sarcoma virus (Ha-ras) and Kirsten murine sarcoma virus (Ki-ras) of the rat (46, 64, 70). A third class, N-ras, is present in the DNA of leukemias, sarcomas, a neuroblastoma and several other human tumor cells (11, 54, 70, 97, 127). However, N-ras has not been found to be retrovirus-associated (11).

The protein products of oncogenic Ha-ras and Ki-ras, designated p21-ras, are similar proteins with molecular weights of 21,000 daltons (10, 54). The oncogenic proteins of mammalian ras genes are localized on the inner face of the plasma membrane. They bind with GTP and may function as a coupling factor (54).

It has been shown that N-myc, a cellular gene homologous to the c-myc proto-oncogene, cooperates with ras oncogenes in the tumorigenic conversion of normal embryonic fibroblasts (130). In another study it has been shown that rat embryo fibroblasts undergo transformation when co-transfected with active myc and Ha-ras oncogenes. However, transformation was not accomplished in rat embryo fibroblasts by either myc or ras alone (70.)

One of the purposes of this study was to demonstrate the induction of alpha interferon mRNA in Sendai virus-induced Namalva cells and to follow the level of alpha interferon mRNA synthesis at the transcriptional level. Efforts were made to correlate levels of alpha interferon mRNA with levels of alpha interferon synthesized by induced Namalva cells.

Another purpose of this study concerned the expression of oncogenes by Namalva cells. It was shown that other human lymphoblastoid cells produce oncogenes, e.g., the c-myc oncogene, as expressed by Daudi cells. This human lymphoblastoid cell line, like the Namalva line, also originated from a Burkitt lymphoma but, upon induction by Sendai virus, produces much lower levels of interferon than Namalva cells. Therefore, it is of importance to determine whether the Namalva cell line expresses the c-myc oncogene and, if it does, to what degree compared to the Daudi line.

The cellular oncogene, c-Ha-*ras*, will also be studied as part of this project. The rationale for this is that c-*myc* and c-Ha-*ras* oncogenes have been shown to act synergistically in tumorigenesis (70, 130), a possibility which would make Namalva cell interferon undesirable for clinical use.

CHAPTER II

MATERIALS AND METHODS

Materials

Cells

Namalva cells and human leukocytes were obtained from the interferon laboratory at the Wadley Institutes of Molecular Medicine, Dallas, Texas, while cells called U cells, Utrecht human amnion cell line, were obtained from Dr. Kari Cantell, Helsinki, Finland. The cell lines; Daudi, human Burkitt lymphoma; BALB/c 3T3, mouse fibroblast; PLC/PRF/5, human hepatoma; and H35, a rat hepatoma cell line, were purchased from the American Type Culture Collection, Rockville, Maryland.

Interferon

Human leukocyte interferon was obtained from the interferon laboratory of Wadley Institutes of Molecular Medicine.

Virus

Sendai virus and vesicular stomatitis virus were obtained from the Wadley interferon laboratory.

Growth Media and Supplements

Roswell Park Memorial Institute 1640 medium, minimal essential medium, minimal essential medium with Earle's

salts and Dulbecco's modified Eagle's medium, L-glutamine, fetal bovine serum, and calf serum were purchased from GIBCO Laboratories, Grand Island, New York. Yeast extract and tryptone were purchased from Difco Laboratories, Detroit, Michigan. Ampicillin, chloramphenicol and cyclohexamide were purchased from the Sigma Company, St. Louis, Missouri. Gentamicin (60 mg per ml) was purchased from Irvine Scientific of Santa Ana, California.

Enzymes

The deoxyribonucleic acid (DNA) polymerase I was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Indiana. Large fragment of polymerase (Klenow fragment) was purchased from DuPont NEN Products, Boston, Massachusetts. Proteinase K was purchased from Bethesda Research Laboratory, Inc., Gaithersburg, Maryland and polynucleotide kinase was purchased from Pharmacia P-L Biochemicals Inc., Piscataway, New Jersey. The deoxyribonucleic acid degrading enzyme (DNase-1) was purchased from Worthington Biochemicals, Freehold, New Jersey while lysozyme was purchased from the Sigma Company.

Probes

The synthetic oligomer 5' TTCGCAGTTTCAC 3', an 18S ribosomal probe (117), single-stranded M13mp11-IFN-α WA DNA (43), double-stranded M13mp11-IFN-αWA DNA (116), and pDF8, a subclone of 18S rat ribosomal DNA in pBR322 (42) were

obtained from the Department of Molecular Genetics, Wadley Institutes of Molecular Medicine. The synthetic oligomer (IFN primer), 5' CAGCCAGGATGGAGTCC 3' was purchased from Biologicals, Toronto, Canada. The plasmid pHSR-1 (in *E. coli* HB101) containing the human cellular *myc* gene (c-*myc*) subcloned into pBR322 (2) and the plasmid pT24-C3 (in *E. coli* C-600) containing c-Ha-*ras*, the human bladder oncogene (94), were obtained from the American Type Culture Collection, Rockville, Maryland.

Reagents

All chemicals were of reagent grade quality or better and were used without further purification unless otherwise indicated. The following reagents were purchased from the Fisher Company, Pittsburgh, Pennsylvania: sucrose, acrylamide, sodium chloride, sodium citrate, sodium acetate, 2-mercaptoethanol, acetic acid, magnesium chloride, boric acid, dimethyl sulfoxide, methylene blue N.F., formaldehyde, chloroform, and formamide.

The following chemicals were purchased from the Sigma Company: Triton X-100, acetic anhydride, triethanolamine, Tris [Hydroxymethyl] aminomethane, (pH 7.4, 7.5, and 8.0), Sephadex G-75, sucrose, ficoll, diethyl pyrocarbonate, ethidium bromide, bromphenol blue, albumin, bovine serum albumin (bovine fraction V), polyvinyl pyrrolidone, salmon sperm DNA, and 3-[N-morpholino]propanesulfonic acid.

Sodium pyrophosphate was purchased from Matheson Coleman and Bell, Norwood, Ohio. Guanidine thiocyanate was purchased from Fluka AG Chemische Fabrik, Bucks, Switzerland. Cesium chloride (Biological Grade) and Tris, ultra pure, were purchased from Schwarz/Mann, Spring Valley, New York. Disodium ethylenediaminetetraacetic acid and glyoxal (40 percent, w/v, in water) were purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin. Ammonium persulfate and N,N,N',N'-tetramethylethylenediamine were purchased from Bio-Rad, Richmond, California. The following chemicals were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Maryland: sucrose, phenol, N,N'-methylene- bis-acrylamide, and ultrapure agarose. The oligo (dt) celluloses, type 2 and type 3, were purchased from Collaborative Research, Inc., Lexington, Massachusetts. Seakem ME agarose was purchased from FMC Corporation, Marine Colloids Division, Rockland, Maine. Xylene cyanol (technical grade) was purchased from Eastman Organic Chemics, Rochester, New York. Gulf charcoal starter was purchased from Gulf Lite, Houston, Texas. Bacto crystal violet (Gram) was purchased from Difco Laboratories, Detroit, Michigan. Acid fuchsin was purchased from Allied Chemical, Morristown, New Jersey. Methylmercuric hydroxide was purchased from Alpha Products, Danvers, Massachusetts. All isotopes were purchased from DuPont NEN Products, Boston, Massachusetts. The dATP, dTTP, dGTP, and dCTP were

purchsed from P-L Biochemicals, Inc., Milwaukee, Wisconsin. Transfer RNA (from brewer's yeast) was purchased from Boehringer Mannheim Biochemicals. Sodium dodecyl sulfate (especially pure) was purchased from Biochemical Company BDH Chemical LTD, Poole, England.

Equipment

The horizontal system for submerged gel electrophoresis Model H5, baby gel apparatus Model H6, and Hybri-dot manifold Number 1050MM were purchased from BRL, Inc., Gaithersburg, Maryland. The vertical gel apparatus Model EC-470 was from E-C Corporation, St. Petersburg, Florida. The Milli-Q-Water purification system was from Millipore Company, Bedford, Massachusetts. The carbon dioxide incubator was from National, Heinicke Company, Portland, Oregon and the vacuum oven, Model 5851, from National Appliance Company, Portland, Oregon. A roll-in incubator was purchased from Belco Biotechnology, Vineland, New Jersey and a Chromato-vue Transilluminator, Model TS-15, from Ultra-violet Products, Inc., San Gabriel, California. The light box was purchased from General Electric, Milwaukee, Wisconsin and the Polaroid MP-4 Land camera from Polaroid, Cambridge, Massachusetts. A fluorescence spectrophotometer, Model 650-10S, was obtained from Perkin-Elmer, Norwalk, Connecticut, the Beckman Model DU-8 spectrophotometer from Beckman Company, Irvine, California, and the Beckman centrifuge Model J2-21 and Beckman ultracentrifuge Model L8-

70 were from Beckman Company, Palo Alto, California. The IEC-DPR-6000 centrifuge was from International Equipment Company, Needham Heights, Massachusetts. The Eppendorf centrifuge Model 5414 was from Brinkmann Instruments, Inc., Westbury, New York, the Sorvall ultracentrifuge, Model OTD65B was from DuPont Company, Newtown, Connecticut, a Kika-Werk Ultra-Turrax homogenizer, Model SDT, was from Tekamar Company, Cincinatti, Ohio, and liquid scintillation counter, Model LS7500, was from Beckman Scientific Instrument Division, Irvine, California. A Bio-dot microfiltration apparatus was purchased from Bio-Rad Laboratory, Richmond, California, and the Stabil-therm Gravity Oven incubator, Model OV-12A, was from Blue M Electric Company, Blue Island, Illinois. The controlled environment incubator shaker was obtained from New Brunswick Scientific, Edison, New Jersey and the tissue grinder from Fisher Company. A transidyne scanning densitometer, Model 2995, was bought from Transidyne General Company, Ann Arbor, Michigan. The Bright Line American optical hemocytometer and inverted microscope Model 1810 were from American Optical Corporation, Buffalo, New York while the Lab-line Digimatic Colony Counter from Lab-Line Instrument, Inc., Melrose, Illinois.

Other Materials Used

Kodak X-O Mat AR film, 20 x 25 cm (8 x 10 inches), and Kodak Xomatic regular intensifying screen were from Eastman

Kodak Company, Rochester, New York. The DuPont Cronex lightning-plus intensifying screen was from Southwest & Johnson X-Ray Company, Dallas, Texas. A heat sealer Counter Craft Deluxe Seal-N-Save, Model 259-6590-01, was obtained from Sears Roebuck Company, Dallas, Texas. Kapak Scotchpak Heat Sealable Pouches were obtained from Kapak Corporation, St. Louis, Missouri while the BA85 pure nitrocellulose paper, 0.45 µm pore, and Quick-Blot kits were purchased from Schleicher and Schuell, Keen, New Hampshire. The 3MM Whatman Paper was purchased from Fisher Company, the Polypropylene Econo-Columns used for chromatography were from Bio-Rad Laboratory, Richmond, California and Nalgene disposable filters were from Nalge Company, Rochester, New York. The 51 mm short-skirt screw cap roller bottles with growth area of 1585 square centimeters (cm^2) were obtained from Belco Glass, Inc., Vineland, New Jersey.

Methods

Cell Growth

Namalva cells were grown in the Roswell Park Memorial Institute 1640 medium containing ten percent (v/v) heat-inactivated fetal bovine serum, 292 mg of L-glutamine, and 50 to 75 mg of gentamicin per liter of culture. Cultures were maintained in 25, 75, and 150 cm^2 flasks in an incubator with carbon dioxide in air at 37°C. In experiments in which the cells were induced, the cells were kept in 51 mm short-

skirt, screw cap Belco roller bottles on a rolling unit in the Belco roll-in incubator at 37°C. The incubator contained no circulating carbon dioxide, therefore each time the cell cultures were divided, carbon dioxide (from a tank containing ten percent, v/v, carbon dioxide in air) was applied to the bottles for a few minutes and the bottle caps tightened again. For mass production of cells, the culture volume was increased to a maximum of one liter each in roller bottles. In all cases, the cells were counted and divided every three days and provided with fresh medium. For counting, cells were mixed with 0.4 percent (w/v) trypan blue in normal saline, placed in a hemocytometer chamber, and the unstained, live cells were counted with an inverted microscope.

Induction of Namalva Cells for Interferon Production

To prepare cells for large scale interferon production, cultures were held at 4°C overnight to allow the cells to settle. Sedimented cells were separated from the growth medium by aspirating the supernatant and were resuspended to a density of 4×10^6 cells per ml of fresh medium containing 292 mg of L-glutamine per liter and five percent (v/v) heat-inactivated fetal bovine serum. After the addition of carbon dioxide to each bottle, the cells were primed with 100 units of interferon per milliliter and incubated at 37°C in a roll-in incubator for about two hours (6). Thereafter, the

cells were induced by adding 150 units* of hemagglutinating Sendai virus, a parainfluenza virus commonly used for induction of Namalva cells, per ml of culture (105, 109). After addition of carbon dioxide, the cultures were incubated as described before for a period of 24 hours. In each experiment, sequential cell samples were taken from the culture, centrifuged 10 to 15 minutes at 900 X g and the supernatants assayed for interferon activity.

Interferon Assay (Plaque Reduction)

Plaque reduction assays were performed using U (Utrecht) amnion cells challenged with vesicular stomatitis virus (VSV) (71). Briefly, U amnion cells in minimal essential medium with Earle's salts (GIBCO Laboratories, Grand Island, New York) containing ten percent (v/v) fetal bovine serum were seeded in 6-cm tissue culture dishes on day one and were incubated at 37°C for two days or until the cell monolayers became confluent. The medium was aspirated on day three and replaced with fresh minimal essential medium containing five percent fetal bovine serum.

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*"Two-fold dilutions (0.25 ml) of test virus were made in normal saline. To each dilution, 0.25 ml of a 0.5% suspension of chicken red-blood cells was added, and the cells allowed to settle. That pattern which showed partial agglutination was taken as the end point and it was read by interpolation if necessary" (56).

The samples were diluted, added to the cells, and incubated overnight. On day four, a sufficient quantity of vesicular stomatitis virus (sufficient to produce approximately 100 plaques in the virus control plates) was added to the plates and these were then incubated for two hours at 37°C. After this, the virus suspension was aspirated and the plates were overlain with 0.6 percent (w/v) agar in minimal essential medium and incubated at 37°C overnight. The next day (day five), the cells were stained with crystal violet to facilitate visualization and the plaques counted using a Lab-Line Digimatic Colony Counter. The interferon was titrated by comparison to an NIH reference standard α-interferon (IFN-α).

Ribonucleic Acid (RNA) Isolation

Special care was taken to protect RNA from extraneous RNase. Gloves were worn throughout all experiments and all glassware was heated to 180°C for 16 hours while disposable plastic dishes were used when possible. All solutions used were filtered through 0.22 or 0.45 µm Nalgene disposable filters to prevent bacterial contamination.

For isolation of total RNA, samples containing approximately 4 to 20 X 10^8 cells were centrifuged at 900 X g for 10 to 15 minutes. The supernatants were saved for interferon assay and the pellets resuspended in 13.6 ml of guanidine thiocyanate solution (4 M in guanidine thiocyanate and 0.1 M in Tris at pH 7.4) and 4 ml of 2-mercaptoethanol.

They were then homogenized using a Kika-Werk Ultra-Turrax homogenizer model SDT and layered over a 10 ml cushion of cesium chloride (refractive index of 1.40) in sodium chloride-sodium citrate (SSC) solution (Appendix, #le). The cells were centrifuged at 146,100 X g at 15°C for 20 to 22 hours and the supernatants decanted carefully. The pellets were then rinsed with oligo (dt) binding buffer (Appendix, #2) to remove DNA. The RNA pellets were either dissolved in water and stored at -80°C or 2.5 volumes of ethanol, plus sufficient sodium acetate to give a final strength of 0.24 M, were added and the mixture allowed to stand at -80°C overnight. In either case, the RNA recovered was measured with a Beckman DU-8 scanning spectrophotometer or a Perkin-Elmer fluorescence spectrophotometer. The RNA pellets were disolved in water and stored at -80°C or lyophilized and stored at -80°C.

Isolation of Poly(A)+ mRNA

The isolation of mRNA from eucaryotic cells has been previously described (82, 113). Samples of RNA from induced and uninduced Namalva cells were further processed and poly(A)$^{+}$ mRNA isolated. An oligo (dt) column was prepared using either 0.5 g oligo (dt) cellulose type 2 or type 3 matrix from Collaborative Research Inc. The total RNA concentration was adjusted to 1 mg per ml of water and 372 µg of ethylenediaminetetraacetic acid (EDTA) pH 8.0

were added for each mg of RNA in the solution. Typically two milligrams of this RNA solution were used for poly(A)$^+$ mRNA isolation and was incubated at 70°C for five minutes and then cooled in an ice bath. Oligo (dt) binding buffer (Appendix, #2) was added to the RNA and the final concentration of RNA adjusted to 250 µg per ml of buffer. The RNA sample was then applied to the column which had been pre-equilibrated with binding buffer and recycled five times before washing with the same buffer. Poly(A)$^+$ mRNA was eluted from the column with elution buffer (Appendix, #3). To the eluted RNA were added 2.5 volumes of ethanol plus sufficient sodium acetate buffered at pH 5.5 to give a final strength of 0.2 M and the mixture held at -80°C overnight. The precipitate was centrifuged at 7,000 to 27,000 X g for 15 minutes and the supernatant discarded. The RNA pellet was then resuspended in water and after addition of ethidium bromide, the concentration was measured by fluorescence at the exciting wavelength of 525 nm and the emission wavelength of 600 nm.

Gel Electrophoresis

The RNA or DNA sample was resolved by electrophoresis. Loading buffer was added to the RNA or DNA samples before their application to the gel. Urea or sucrose in solid form, or as much as a 1/10 volume of a loading buffer was typically used. Two types of loading buffers were used: (Appendix,#4a and #4b). The following gel systems were used:

a. Agarose gel electrophoresis. The RNA samples and DNA markers were resolved in one percent (w/v) agarose gel hydrated with Peacock's buffer (Appendix, #5a). Freshly made Peacock's buffer was also used for electrophoresis. The gels, 12.2 cm x 17.7 cm, were run for approximately one hour at 300 volts in the vertical apparatus at approximately 25°C and for about one hour at 100 volts in the baby gel apparatus for 5.1 cm x 7.6 cm gels. They were then stained with ethidium bromide (1 ug per ml of water) for 15 to 30 minutes and observed under a chromato-vue transilluminator model TS-15 where pictures could be made with the Polaroid MP.4 Land camera.

b. Electrophoresis of RNA samples treated with methylmercuric hydroxide. Aqueous methylmercuric hydroxide (1 M) was added to the RNA samples to a final concentration of 0.1 M. The samples were resolved by electrophoresis and the gels stained as described for the agarose gel electrophoresis.

c. Electrophoresis of RNA and DNA samples treated with glyoxal. Both the RNA and DNA samples were glyoxalated in dimethyl sulfoxide (Appendix, #6) at 50°C for one hour. After glyoxalation, the samples were rapidly chilled and placed on 1.5 percent (w/v) agarose gels (11 cm x 13.5 cm) in a 10 mM sodium phosphate buffer at pH 6.5 in a horizontal apparatus. The samples were resolved by electrophoresis overnight with 40 volts at 4°C in a 10 mM sodium phosphate electrophoresis

buffer at pH 6.5. They were stained with 0.2 percent (w/v) methylene blue (Appendix, #7) for about 20 minutes and destained in water for one or two days or until destaining was complete. The nucleic acid bands were then observed under ultraviolet radiation and the gels were photographed as previously described.

d. Electrophoresis of RNA samples treated with formaldehyde. The method of Maniatis et al. was used with minor modifications (81). In brief, RNA samples were denatured in the mixture described in Appendix #8. The denatured samples were incubated at 55°C for 15 minutes, chilled, and placed on 1.2 percent (w/v) agarose gels in 3-[N-morpholino]propanesulfonic acid (MOPS) buffer and formaldehyde (Appendix, #8). The samples were resolved by electrophoresis with 200 volts in the horizontal apparatus for approximately two hours in MOPS buffer (20 mM MOPS, 5 mM sodium acetate and 0.01 mM EDTA in water) (Appendix, #8) at room temperature. The gels (11 cm x 13.5 cm) were then stained with 0.2 percent (w/v) methylene blue (Appendix, #7) and photographed as previously described.

e. Composite gel electrophoresis. The RNA samples were resolved by electrophoresis on composite gels (Appendix, #9) with 200 volts for two hours at room temperature in the vertical apparatus. The gels were stained with ethidium bromide (1 µg per ml of water) for one-half hour and photographed as described before.

f. Paper electrophoresis for mononucleotide separation. In order to determine the amount of labeled nucleotides incorporated into the oligomer probe, paper electrophoresis was performed as follows:

Paper preparation: 3MM papers were cut to 20 cm x 34 cm and folded three cm from each end on the 20 cm side. A pencil line was drawn across the paper 2.5 to 3 cm from one folded end and the site of each sample application was identified by pencil at intervals of approximately 2.5 cm on this line.

Electrophoresis equipment: The electrode buffer was aqueous five percent acetic acid solution adjusted to pH 3.5 with ammonium hydroxide and added to a depth of three-quarters of one inch in the electrode wells. Buffer chambers and paper support platform were covered with Gulf Lite charcoal starter to approximately one-half inch over the paper platform. Buffer droplets were swept from the paper support platform into the electrode chambers with a piece of 3MM paper.

Loading samples: Papers were sprayed with electrode buffer in a fume hood until thoroughly wet. When dripping stopped, they were hung from the bench edge with the sample line at the bottom. Both sides of the sample loading area were blotted with paper towels. An acid fuchsin marker was spotted on the pencil line between sample marks.
The papers were held at the bottom to provide tension.

Approximately 1 to 2 µl of each sample were spotted onto the papers which were then submerged so that the folded ends reached into the electrode buffer and all bubbles on the papers were removed.

Electrophoresis: Samples were resolved by electrophoresis at 500 or more volts (approximately 30 mA) for approximately one hour at room temperature. Nucleotides and inorganic phosphate migrated toward the anode and the acid fuchsin spot migrated approximately the same distance as did the inorganic phosphate. Papers were removed and air-dried under a fume hood with a hot air dryer and autoradiographed.

Transfer of RNA onto Nitrocellulose Paper (Northern Transfer)

The RNA samples were transferred to nitrocellulose paper in the same manner as described for transfer of DNA (103). A baking dish (18.5 cm x 31.5 cm) was half-filled with SSC (Appendix, #1f). Either a piece of glass (20 cm x 20 cm) was placed on top and across the dish or a sponge (9 cm x 17 cm) was put in the dish. In either case, a piece of Whatman 3MM paper was placed on the glass or sponge and each side of the paper was folded and inserted into the transfer solution. The gel was placed on top of the 3MM paper, followed by a piece of BA85 (0.45 µm pore size) nitrocellulose paper and then two pieces of 3MM paper the same size as the gel. A stack of paper towels was put on

top of the 3MM paper and a weight was placed on top of the towels to maintain contact between the nitrocellulose paper and gel. The transfer set was left overnight. On the following day, the nitrocellulose paper was heated for two hours at 80°C in a vacuum oven and the samples were either used immediately for the hybridization experiments or stored in a desiccator at room temperature.

Labeling the Probes

a. End labeling (5'). The oligomeric probes were labeled in a reaction mixture (Appendix, #10) and incubated at 37°C for one-half hour. The percentage of incorporation was estimated by using paper electrophoresis as described above.

b. Primer extension. Single-stranded M13mp11-IFN-αWA DNA was used as a template (43). The reaction mixture containing a DNA probe (Appendix, #11) was incubated at 37°C for 15 minutes and the DNA was hybridized to the RNA samples.

c. Nick translation. Double-stranded M13mp11-IFN-αWA DNA (116) was nick-translated. In the first experiment using an interferon probe and the dot blot technique, (p. 78 of Results section) the total volume of the reaction mixture was 32 µl (Appendix, #12a). In the other interferon hybridization process, the reaction volumes were held to 25 µl by modifying the concentration of some of the ingredients

(Appendix, #12b). The reaction mixture was incubated at 12 to 13°C in a water bath for about one and one-half hours and then 2 µl of 0.5 M EDTA were added to stop the reaction. After increasing the volume to 50 µl by addition of Tris-EDTA solution (Appendix, #13), the sample was extracted with a phenol-chloroform solution. Sucrose was added to the sample to facilitate loading onto a column of Sephadex G-75. A 2 ml siliconized pipet plugged with glass wool was used as a column. Two-drop fractions (50 to 90 µl) were collected and radioactivity counted using a Beckman liquid scintillation counter. The fractions of the first peak were combined and, after calculation of specific activity, the labeled DNA was used for hybridization.

The pHSR-1 plasmid DNA containing the c-*myc* gene (2) and pT24-C3 plasmid DNA containing the human bladder oncogene c-Ha-*ras* (94) were also labeled by nick-translation. When needed, approximately 0.5 to 1 µg of DNA was nick-translated as described.

Different Hybridization Procedures of Namalva Cell RNAs with Ribosomal DNA and Interferon Probes

1. Hybridization of RNA from induced and uninduced Namalva cells with ribosomal DNA probe (17mer synthetic oligomer)

   a. Dot blot hybridization. The RNAs from Namalva cells were spotted directly onto nitrocellulose papers or onto the papers after Northern transfer from formaldehyde gels. In either case, the filters

were heated at 80°C in a vacuum oven for approximately two hours. The heated filters were placed in plastic bags (Kapak Scotchpak heat sealable pouches) and closed with a heat sealer. The hybridization buffer (Appendix, #14a) was injected into the bags by syringe, bubbles were removed, and the bags sealed. End-labeled synthetic oligomer, 5' TTCGCAGTTTCAC 3' (117), was added and the bags sealed again. They were then placed in an incubator (Blue M Electric Company, Model OV-12A equipped with a homemade rotor) at 37°C overnight for RNA hybridization. The filters were then washed with SSC (Appendix, #1d) two to three times at 37°C for about 15 minutes each time.

b. Northern hybridization. The RNA was resolved by electrophoresis on formaldehyde gels and transferred onto nitrocellulose papers. The RNA solution was applied as dots on the edges of the nitrocellulose papers before heating. The procedure for hybridization of RNA and washing of the filters was as described for dob blot hybridization above.

2. Hybridization of Namalva cell RNA with extended primer (IFN probe).

Solutions of total RNA and poly(A)+ mRNA from induced

and uninduced Namalva cells were applied as dots onto nitrocellulose papers and the papers were heated for two hours. The RNA samples were prehybridized in Denhardt's Solution (Appendix, #14b) at about 40°C for four to six hours and hybridized overnight at the same temperature after injection of the ^{32}P-labeled extended primer probe. After hybridization, the papers were washed three to four times with SSC (Appendix, #1c) and 0.1 percent (w/v) sodium dodecyl sulfate (SDS) at room temperature for about five minutes each. This was followed by two washes with SSC (Appendix, #1a) and 0.1 percent (w/v) SDS for a total of two hours at the hybridization temperature.

3. Hybridization of Poly(A)+ mRNA of induced and uninduced Namalva cells with interferon (IFN) probe (IFN-α WA).

a. Dot blot hybridization and quantitative analysis of IFN-α mRNA level. The Poly(A)+ mRNA from different cell samples in 1.5 µg amounts was diluted to 50 µl with sterile water. Then, 150 µl of 6.15 M formaldehyde in SSC (Appendix, #1e) was added to each sample to make a total volume of 200 µl. Samples were incubated at 65°C for 15 minutes and were cooled in an ice bath. Nitrocellulose papers were soaked in water and then in SSC prior to placement in a 96-well (8 x 12 wells) dotting device (either the Hybridot manifold from Bethesda Research Laboratories or the Bio-Dot apparatus from

BioRad). The apparatus was connected to a vacuum line and different quantities of each sample were applied. These were then washed with 0.2 ml of SSC (Appendix, #1e). After heating the papers at 80°C for two hours they were sealed in plastic bags as previously described and the RNA samples prehybridized in Denhardt's solution (Appendix, #14c) for about six hours at 42°C in a rotating incubator. After prehybridization, nick-translated interferon probe (116) was placed in boiling water for ten minutes and cooled in an ice bath before injecting into the bags. The RNA samples were hybridized overnight at 42°C. After hybridization, the filters were washed three times in SSC (Appendix, #1c) and 0.1 percent (w/v) SDS at room temperature for 10 to 15 minutes each and two times in SSC (Appendix, #1a) and 0.1 percent (w/v) SDS in water at 50°C for one-half hour each. The filters were then dried and autoradiographed.

The density of the hybridized poly A^{+}mRNA dots on the radiogram was measured and graphed using a Transidyne scanning densitometer.

b. Northern hybridization. The Namalva cell poly$(A)^{+}$ mRNA and ^{32}-P labeled λ DNA markers were glyoxalated, resolved by electrophoresis and

transferred onto nitrocellulose papers. The papers were heated and the samples were prehybridized in Denhardt's solution (Appendix, #14d) at 37°C with rotation for several hours. Hybridization buffer was basically the same as described by Wahl et al. (125) with only slight modifications (Appendix, #14d). After prehybridization, nick-translated interferon probe (116) was heat denatured and injected into the hybridization bags. The samples were hybridized overnight at 37°C as previously described. After hybridization, the filters were washed overnight at 37°C in the same buffer minus salmon sperm DNA and were washed again in SSC (Appendix, #1d) and 0.1 percent (w/v) SDS for six to eight hours at 37°C.

4. Hybridization of Namalva cell RNA with 18S ribosomal DNA.

The RNA of Namalva cells and a ^{32}P-labeled λ DNA marker were glyoxalated and resolved by electrophoresis on 1.5 percent (w/v) agarose in sodium phosphate buffer as described earlier. The RNA was then transferred onto nitrocellulose papers and held overnight at room temperature. After transfer, the papers were heated and the samples were hybridized with nick-translated pDF8, a subclone of 18S rat ribosomal DNA in pBR322 (42). The filters were washed and autoradiographed. Conditions for

hybridization and washing of the filters were the same as described in section 3-b.

5. Quick-blot hybridization of Namalva, Daudi, PLC/PRF/5, BALB/c 3T3 and H35 mRNA with c-*myc* probe

a. Isolation of plasmid DNA containing the c-*myc* oncogene. Amplification and isolation of plasmid DNA was basically as described by Maniatis *et al*. (81). The plasmid pHSR-1 carries a 9 kilobase (kb) EcoRI-HindIII fragment isolated from a human c-*myc* clone in bacteriophage and subcloned into pBR322 which has a total size of 13.3 kb. *Escherichia coli* HB 101, containing this plasmid, was grown in Luria broth (Appendix, #15) in a shaker incubator at 37°C. Ampicillin, 25 µg per ml was added to prevent growth of contaminating bacteria since the bacteria containing pHSR-1 were ampicillin resistant. When the culture reached an optical density at a wavelength of 600 nm of 0.6 to 0.8, chloramphenicol to a final concentration of 100 µg per ml was added to stop protein synthesis and amplify plasmid DNA synthesis. The culture was incubated at 37°C in a shaker overnight and on the next day the cells were centrifuged at 9,000 X g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 20 ml of lysing

buffer (Appendix, #16) per liter of original culture. The cell suspension was divided into two tubes and lysozyme solution was added to each tube to make a final concentration of one mg per ml of lysing buffer. The tubes were kept on ice for ten minutes and 2 ml of 0.5 M EDTA (pH 7.6) were added to each tube. The tubes were inverted several times and were kept on ice for five minutes. Next, 7.5 ml of Triton-X-100 mixture (Appendix, #17) was added to each tube and they were mixed by inverting one or two times and were kept on ice for ten minutes. The samples were centrifuged at 48,000 X g for two and one-half hours at $0^{o}C$ and the supernatants were saved. For every 7.4 ml of supernatant, 7.2 g of cesium chloride and 0.8 ml of ethidium bromide (5 mg per ml of water) were added to each tube and these centrifuged at 146,000 X g at $18^{o}C$ for 48 hours. After centrifugation, plasmid bands were observed under ultraviolet radiation and the plasmid DNA extracted by the method of Maniatis _et al_. (81). An equal volume of isopropanol was added to the plasmid solution. The samples were mixed and after a few minutes two layers formed in the solution. The upper layer was discarded. This step was repeated several times until the upper layer became clear. The plasmid

DNA was then precipitated with ethanol at -80°C; and centrifuged at 14,000 X g at 0°C for 25 minutes. The pellet was resuspended in 0.3M sodium acetate solution, reprecipitated with ethanol and centrifuged at 14,000 X g for one-half hour. The plasmid DNA was dissolved in SSC (Appendix, #1b) and measured by spectrophotometer at a wavelength of 260 nm. The samples were resolved by electrophoresis in one percent (w/v) agarose in Peacock's buffer (Appendix, #5a).

b. Quick-blot procedure. The "Quick-Blot" technique is based on the immobilization of mRNA from whole cells onto nitrocellulose paper (44). Cells were pelleted and resuspended in 18 ul of Hank's solution containing 50 µg per ml of cycloheximide. To inhibit protein synthesis, 2 ug (in 2 µl) of proteinase K was added to the sample. A kit containing reagents A-D (Appendix, #18) from the Schleicher and Schuell company was used for the quick-blot procedure. Samples were applied onto nitrocellulose paper. Following the dot-blot procedure the paper was washed three times with RNase-free water, followed by three 5-minute washes with 70 percent (v/v) ethanol in water. The paper was then soaked in freshly prepared acetic anhydride solution (Appendix, #19) for ten minutes.

After the last wash, the paper was air dried and kept in a desiccator until needed for the hybridization experiment.

c. Sample preparation and hybridization. The cells used in the Quick-Blot experiment were: Namalva and Daudi (human Burkitt lymphoma cell lines), PLC/PRF/5 (a human hepatoma cell line), BALB/c 3T3 (a mouse fibroblast cell line), and H35 (a rat hepatoma cell line). The Namalva and Daudi cells were grown in Roswell Park Memorial Institute 1640 medium (GIBCO Laboratories) containing ten percent (v/v) heat-inactivated fetal bovine serum. The PLC/PRF/5 cells was grown in minimal essential medium (GIBCO Laboratories) containing ten percent (v/v) heat inactivated fetal bovine serum while BALB/c 3T3 cells were grown in Dulbecco's modified Eagle medium containing ten percent (v/v) heat inactivated calf serum. The H35 cells were grown in minimal essential medium containing five percent (v/v) heat-inactivated fetal bovine serum and five percent (v/v) heat-inactivated calf serum. All cells were grown in an atmosphere of carbon dioxide in air at 37°C. Ribonucleic acid (RNA) isolated from three quantities of Namalva and Daudi cells, 0.25×10^6, 0.5×10^6, and 1×10^6, were treated with

detergent and sodium iodide and applied onto the nitrocellulose paper. Similarly, RNA isolated from three different quantities of PLC/PRF/5, BALB/c3T3 and H35 cells (0.125×10^6, 0.25×10^6 and 0.5×10^6) were also applied onto nitrocellulose papers as described (Appendix, # 18). Hybridization conditions were the same as described above. The probe, pHSR-1, which contained an insert of 9.0 kb carrying the c-*myc* gene (2) was nick-translated. After hybridization, the filters were washed three times in SSC (Appendix, #1e) and 0.1 percent (w/v) SDS, for 15 to 30 minutes each at 42°C. Next, the filters were washed in SSC (Appendix, #1b) and 0.1 percent (w/v) SDS for one hour at 50 to 65°C.

6. Northern hybridization of RNA isolated from Namalva cells, Daudi cells and leukocytes with c-*myc* probe

Samples of 40 µg each of RNA isolated from Namalva cells, Daudi cells and leukocytes were glyoxalated and resolved by electrophoresis on agarose gels. A λ Hind III fragment was glyoxalated and used as a marker. The samples were transferred onto nitrocellulose papers which were heated at 80°C. The samples were treated for hybridization at 42°C as previously described (Section 3-B). The nick-translated c-*myc* DNA, described in section 5 above was again used as a probe. After hybridization, the filters were washed four times for 5 minutes each at room temperature, in SSC

(Appendix, #1c) and 0.1 percent (w/v) SDS and twice for 15 minutes each at 50°C in SSC (Appendix, #1b) and 0.1 percent (w/v) SDS. The nitrocellulose papers were then dried and autoradiographed.

7. Northern hybridization of RNA isolated from Namalva cells, Daudi cells and leukocytes with c-Ha-ras probe

a. Isolation of plasmid DNA containing the c-Ha-ras oncogene. The Escherichia coli C-600 bearing the plasmid pT24-C3 was purchased from the American Type Culture Collection. To construct pT24-C3, a 6.6 kb BamHI fragment containing an Ha-ras oncogene which was isolated from T24 human bladder carcinoma cell line was inserted into the BamHI site of pBR322 (94). The procedure for growth of bacteria and isolation of plasmid DNA was followed as for c-myc, except that chloramphenicol was added to the culture but only after the optical density at 600nm exceeded a value of 0.8.

b. Northern hybridization. The same Northern filter that was used in the c-myc hybridization experiment (section 6) must be used for the ras hybridization. This filter was placed in RNase-free boiling water for ten minutes to remove the c-myc probe (115). Various RNA samples were then hybridized with a nick-translated c-Ha-ras probe.

The filter was washed thereafter as described above and autoradiography was performed as described below.

Autoradiography

Following each hybridization, nitrocellulose papers were washed, dried and wrapped in plastic wraps. The papers were then pressed onto Kodak films with intensifying screens at $-80^{o}C$. After an appropriate time interval, the films were developed and radioactivity measured as previously described.

CHAPTER III

Results

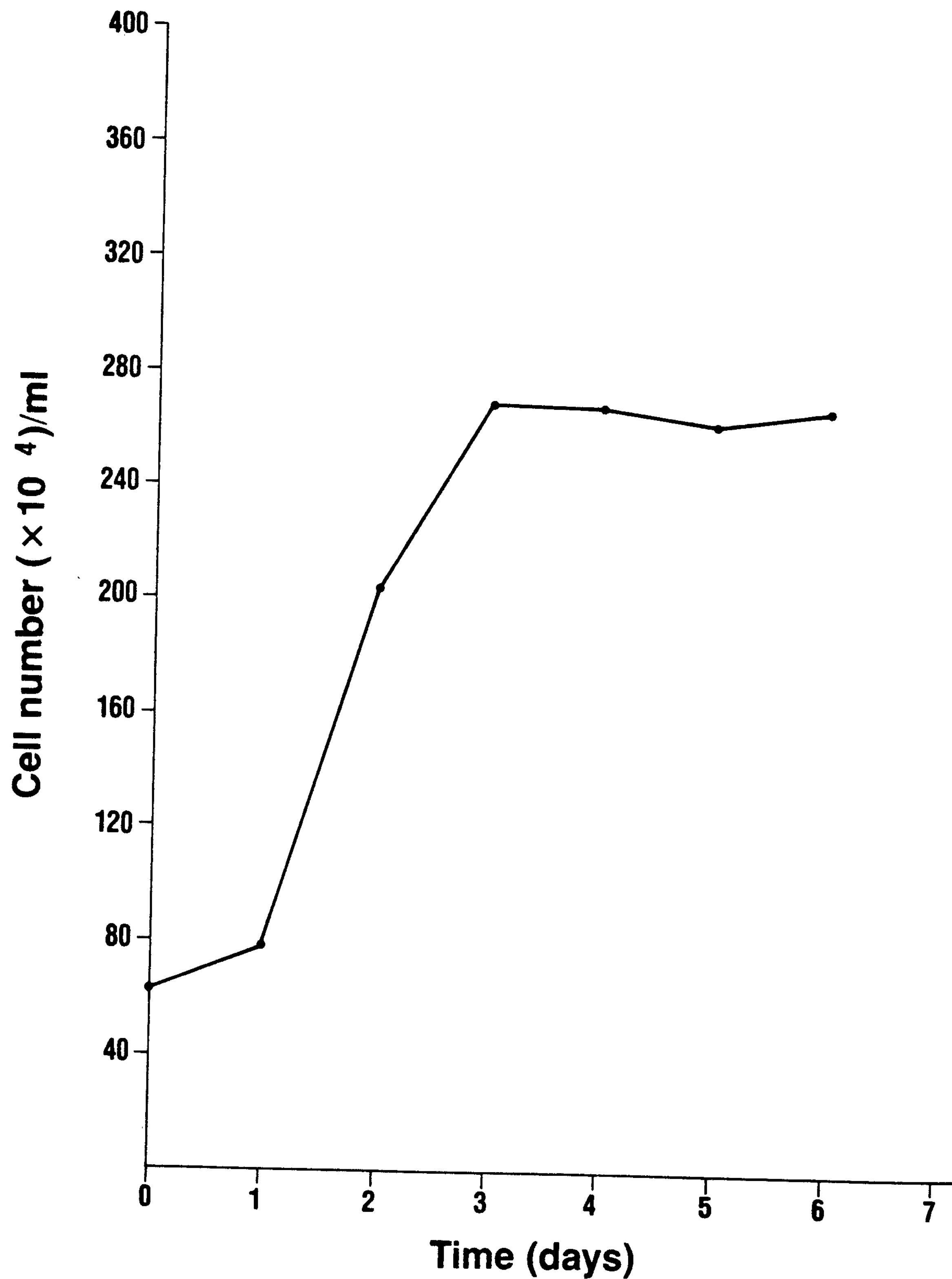
Cell Growth

Namalva cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing ten percent (v/v) heat-inactivated fetal bovine serum and 292 mg of L-glutamine and 50 to 75 mg of gentamicin per liter as described. Samples were taken every day for one week and the cells counted. The number of viable cells per milliliter of medium was plotted against time as shown in Figure 1. After a one day lag phase, the cells started the log phase of growth. This phase lasted about two days (one and one-half doublings). On day three, the pH dropped below 7.0, the cells stopped growing and the number remained almost the same for about four days.

Induction of Interferon in Namalva Cells

Some Namalva cells were induced by Sendai virus as described in Methods. Total RNA was isolated from each induced or uninduced cell sample. For each preparation, two samples were taken for interferon assay, the first sample between four and five hours after induction and the second sample 24 hours after induction. The results of several such measurements are recorded in Table I. The

Fig. 1--Growth curve of Namalva cells. Namalva cells were grown in Roswell Park Memorial Institute 1640 medium containing ten percent (v/v) heat-inactivated fetal bovine serum and 292 mg L-glutamine in an incubator containing five percent (v/v) carbon dioxide in air at 37^{0}C. Culture samples were taken every day, mixed with 0.4 percent (v/v) trypan blue, and viable cells counted using a hemocytometer.



Cell number (×10 4)/ml
400
360
320
280
240
200
160
120
80
40
0
1
2
3
4
5
6
7
Time (days)

TABLE I

INTERFERON AND RNA FROM NAMALVA CELLS INDUCED BY SENDAI VIRUS

Expt. no.	Volume of culture (RPMI)[1] (ml)	IFN, Units per 1 x 10^6 cells (time, hours after induction at 37°C)		RNA (μg/1 x 10^6 cells 5 hrs after induction)[4]
		5 hrs[2]	24 hrs[3]	
1	1000	6,000	7,000	1.76
2	1000	3,500	9,500	1.12
3	800	1,475	2,500	10.78
4	900	1,475	10,250	11.76
5	896	1,168	5,500	6.94
6	1000	833	5,500	4.63

[1]Roswell Park Memorial Institute medium.
[2]Averge 2408; standard error ± 813.
[3]Average 6708; standard error ± 1170.
[4]Average 6.16; standard error ± 1.83.

yield of RNA isolated from induced cells varied from 1 to 11 µg per 1×10^6 cells. In some cultures where the cells were assayed 24 hours after induction, interferon titers reached 10,250 units of interferon per 1×10^6 cells. The lowest titer recorded was 833 units of interferon per 1×10^6 cells in cells induced 4.5 hours before samples were drawn.

Total Cell RNA Evaluation

The 5S, 18S, 28S bands shown in Figure 2 are characteristic of ribosomal RNA and these results indicate that the RNA was not totally degraded by extraction and subsequent manipulation.

The 18S and 28S bands, characteristic of ribosomal RNA, are shown in Figure 3. This experiment also revealed that the RNA was not wholly degraded since 5S bands are usually not seen in this procedure.

The RNA from Namalva cells was also resolved by electrophoresis using the composite gels. Staining these with ethidium bromide revealed the characteristic ribosome bands (Figure 4). The results obtained from the above experiments (Figures 2, 3, 4) indicate that at least a part of the different RNAs isolated, whether denatured before electrophoresis or not, were intact and not degraded.

Fig. 2--Electrophoresis of RNA from uninduced Namalva cells on a one percent (w/v) agarose gel in Peacock's buffer. The gel was stained with ethidium bromide. Lane A contains 17.5 µg, lanes B and C, 7 µg, and lane D, 3.5 µg of RNA. Arrows indicate 5S, 18S, and 28S bands.

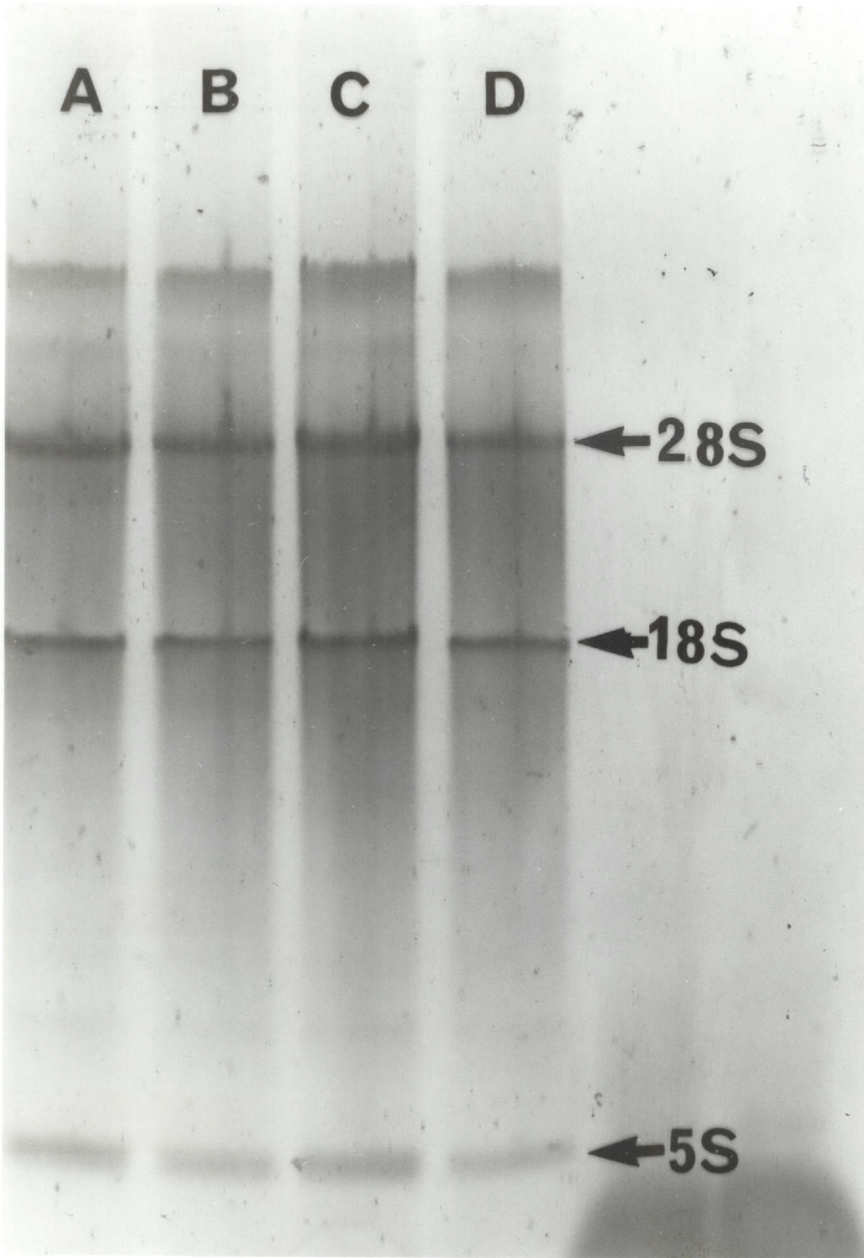

A
B
C
D
28S
18S
5S

Fig. 3--Electrophoresis of 4.24 μg of RNA from Namalva cells on a formaldehyde gel. Gels were stained with methylene blue and destained in water before photographing as described in Methods. The 18S and 28S bands are indicated.

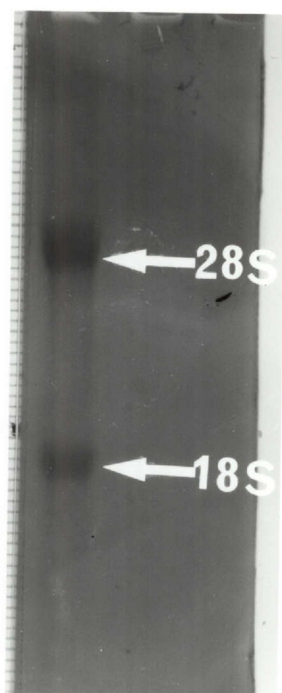
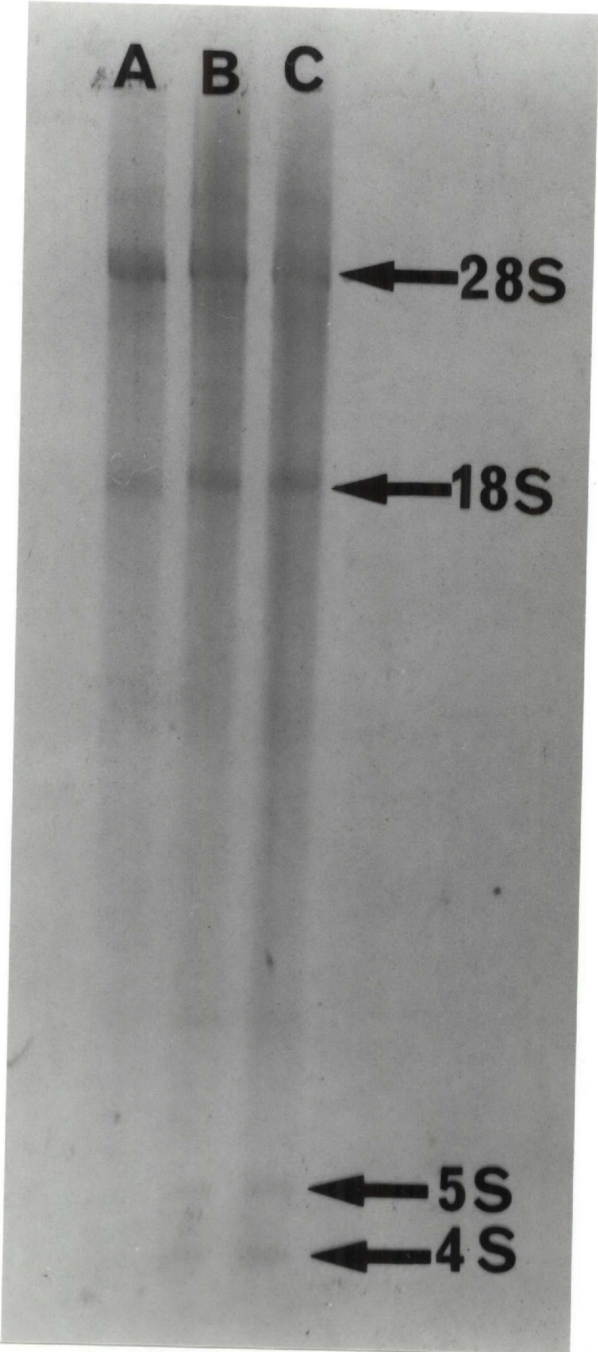

28S
18S

Fig. 4--Electrophoresis of Namalva cell RNA on a composite gel [2 percent (w/v) polyacrylamide, 0.5 percent (w/v) agarose]. The gel was run at 200 volts for two hours and stained with ethidium bromide. The material in lanes A, B and C represents different concentrations of the same sample of RNA. The 4S, 5S, 18S and 28S bands are indicated.


A
B
C
28S
18S
5S
4 S

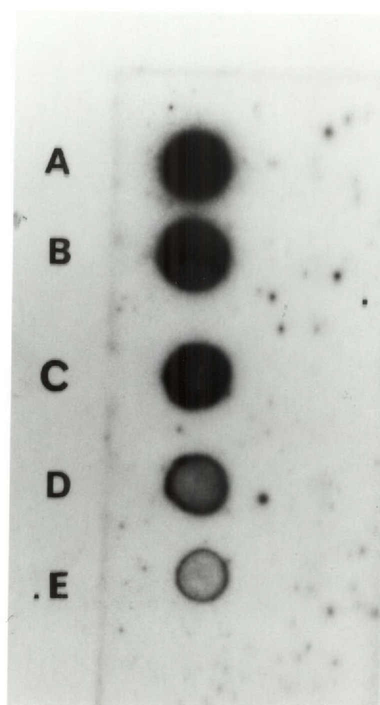
Dot Blot Hybridization of RNA from Namalva Cells with an End-labeled Oligomer Complementary to 18S rRNA

The RNA from Namalva cells was spotted directly onto nitrocellulose papers and hybridized with the oligomeric probe as described in Figure 5. The filters were washed and autoradiographed as described in Methods and the films were developed after 27 hours of exposure. These hybridization experiments revealed that the synthetic oligomeric probe hybridized with the RNA samples (Figure 5). As can be seen in the figure, samples of RNA as small as five ng could be detected by this method.

Northern Hybridization of RNA from Namalva Cells with an End-labeled Oligomer Complementary to 18S Ribosomal RNA

The RNA used in this experiment was from the same batch of cells used in the dot blot hybridization experiments (Figure 5). Namalva cell RNA in amounts of 2.81 and 5.62 µg was resolved by electrophoresis on formaldehyde gels and then transferred onto nitrocellulose paper as described above. After Northern transfer, RNA was spotted directly onto the same nitrocellulose paper as described in Figure 6. The filter was heated for two hours in a vacuum oven and the samples were hybridized with the oligomeric probe. The oligomer (17-mer) was 5' end-labeled with ATP[γ-^{32}P]. The percentage of incorporation was measured by paper electrophoresis and about 50 percent of probe was labeled as

Fig. 5 -- Dot blot hybridization of Namalva cell RNA with end-labeled oligomer complementary to 18S ribosomal RNA. The RNA from Namalva cells was spotted in increasing amounts onto nitrocellulose paper, hybridized with 18S ribosomal probe and the filter was washed and exposed as described in Methods. The filters were autoradiographed and the films were developed after 27 hours. Lanes A, B, C, D, and E contained RNA, 136, 54, 27, 13.6 and 5 ng, respectively.


A
B
C
D
E

seen in Figure 7. The one-hour of autoradiography of the RNA samples revealed the 18S ribosomal RNA bands and RNA dots (Figure 6). The 18S bands were detected in quantities as low as 2.81 µg of RNA. The RNA that was spotted directly onto nitrocellulose paper was detected in quantities as low as 55 ng. Thus the dot blot technique was more sensitive than the Northern blot technique.

Isolation of Poly(A)+ mRNA from Namalva Cell RNA

The Poly(A)+ mRNA was separated from induced and uninduced Namalva cell RNA using an oligo(dt) column. The poly$(A)^+$ mRNA was placed on one percent (w/v) agarose gel in Peacock's buffer (Appendix, #5a) as described in Figure 8. As can be seen from the figure, electrophoresis revealed the 4S, 5S, 18S and 28S bands from RNA and faint bands of 18S from poly$(A)^+$ mRNA lanes A, B, and C. These faint bands were due to contamination with ribosomal RNA since the oligo (dt) column binds only with poly $(A)^+$mRNAs.

Dot Blot Hybridization of Total RNA and Poly$(A)^+$ mRNA of Namalva Cells with Extended Interferon Primer

The RNA and Poly$(A)^+$mRNA from uninduced and induced cells were spotted directly onto nitrocellulose paper as described in Figure 9. The RNA samples were hybridized with extended interferon primer and autoradiographed. The films were developed after 48 hours. No hybridization took

Fig. 6--Northern and dot blot hybridization of RNA from Namalva cells with oligomeric probe complementary to 18S ribosomal RNA. Northern: Lanes A, B, and C contained 5.62, 2.81 and 2.81 µg of RNA. Dots: The material in lanes A, B, and C is RNA spotted in quantities of 5.5, 0.55, and 0.055 µg, respectively. The film was developed after one hour of autoradiographic exposure.

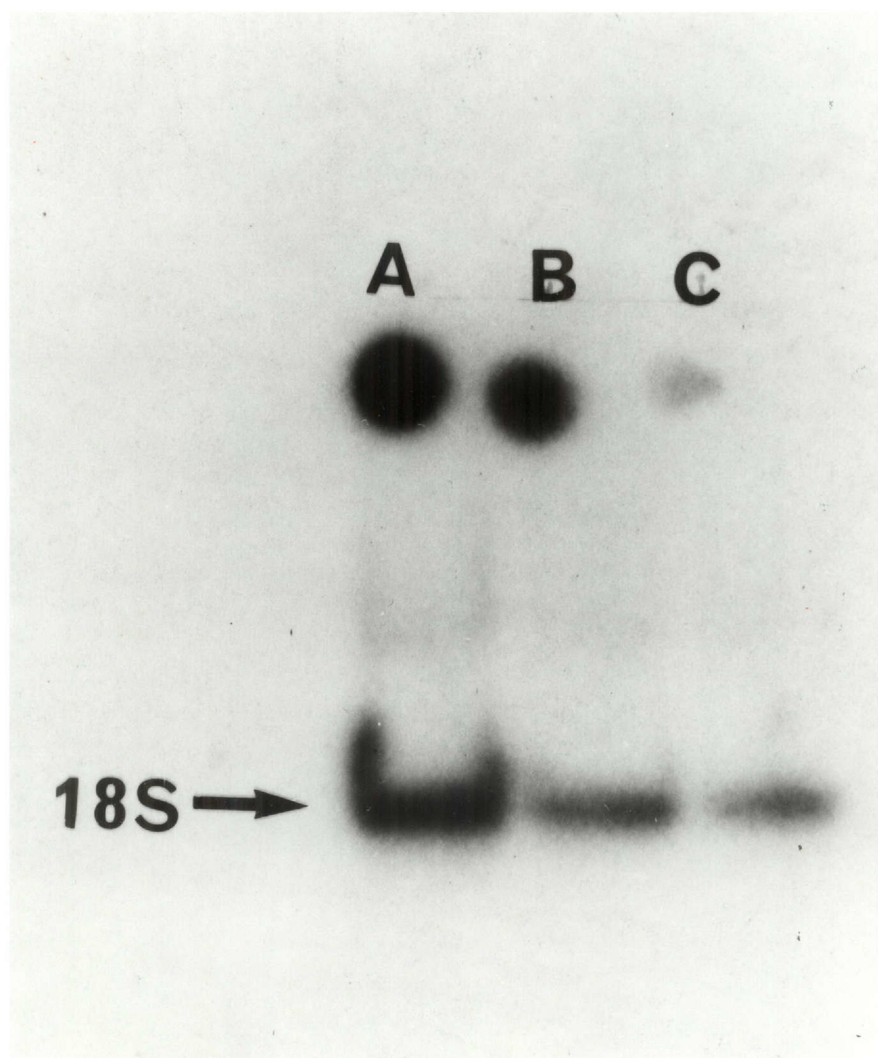

A
B
C
18S

Fig. 7--Paper electrophoresis of end-labeled oligomer. Labeled oligomer (2 µl) was resolved by electrophoresis at 500 volts for one hour as described in Methods. Number 1 is inorganic phosphate, and number 2 is labeled oligomer. The labeled spots between numbers 1 and 2 are ATP. The autoradiography film was developed after one hour of exposure.

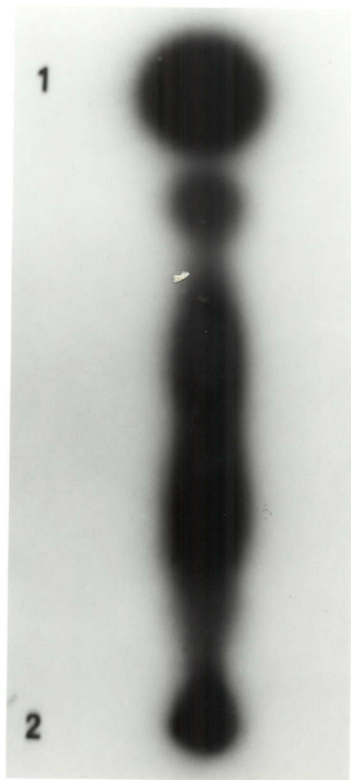

1
2

Fig. 8--Electrophoresis of RNA and poly(A)$^{+}$ mRNA of induced Namalva cells on one percent (w/v) agarose gels in Peacock's buffer. Gels were stained and photographed as described in Methods. Lanes A and B contained 384 ng and lane C, 192 ng of poly (A)$^{+}$mRNA. Lanes D, E, and F contained, 22, 55, and 27.5 µg of RNA, respectively.

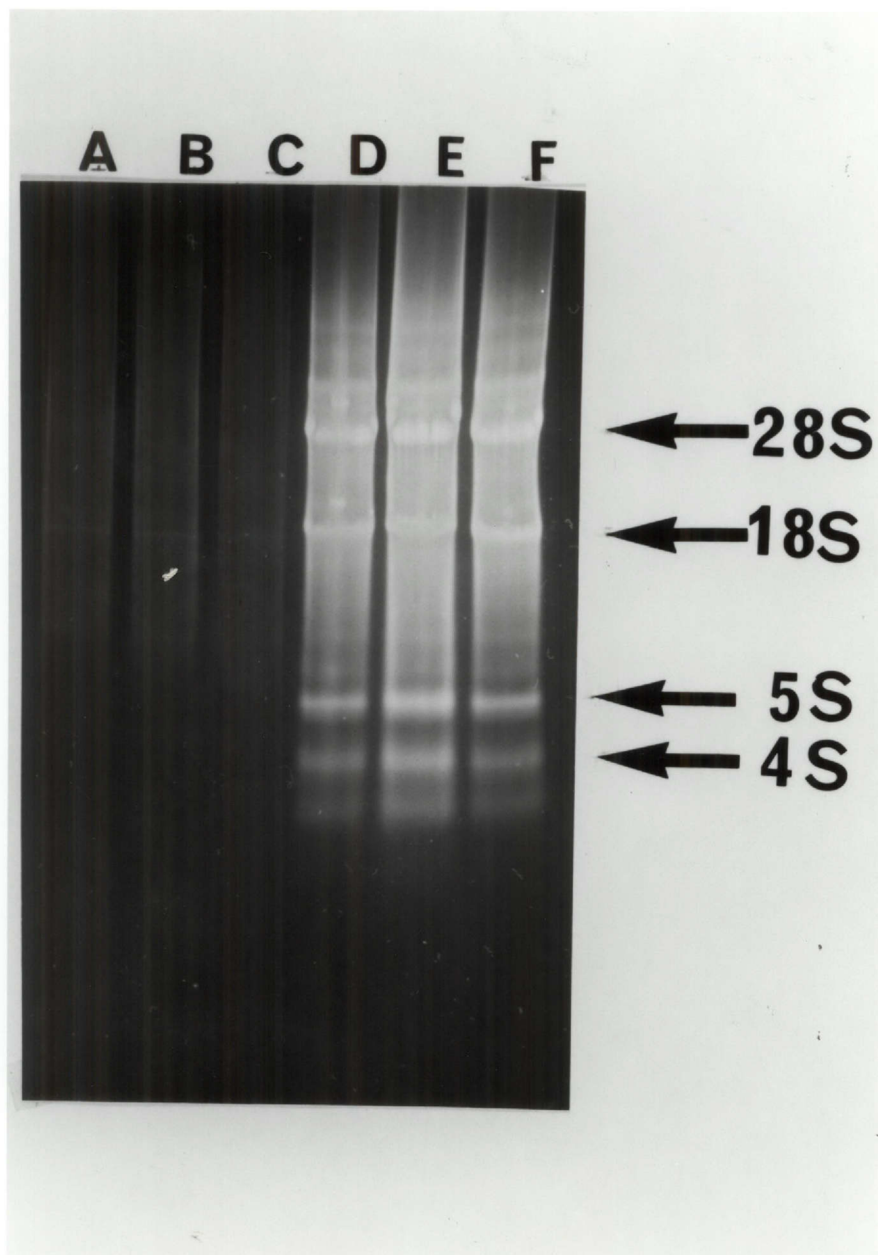
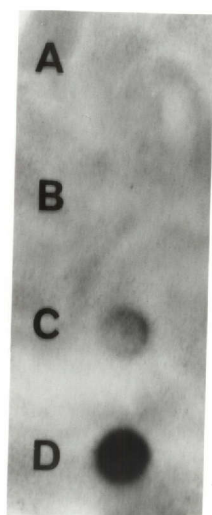

A B C D E F
28S
18S
5S
4S

Fig.9--Dot blot hybridization of RNA from Namalva cells and poly(A)$^+$ mRNA with extended interferon primer. The material in lane A was 8.5 µg of RNA isolated from uninduced cells; B, 7.2 µg of RNA isolated from induced cells; C, 11.7 µg of poly (A)$^+$mRNA, isolated from uninduced cells; and D, 6.8 µg of poly (A)$^+$mRNA, isolated from induced cells.


A
B
C
D

place in total RNA samples but poly(A)$^+$ mRNA, from induced and uninduced cells, hybridized with the probe (Figure 9). Thus, poly(A)$^+$ mRNA was found to be more suitable for hybridization than total cell RNA.

Dot Blot Hybridization of Namalva Cell Poly(A)$^+$ mRNA with Nick-translated M13mp11-IFN-α WA (First Experiment)

a. Culture preparation. A total of 12 X 10^9 cells was used for this experiment. After induction of cells with Sendai virus, five samples were taken at intervals of six hours for RNA isolation and interferon assay. The first sample, zero hour, was taken immediately after addition of the inducer and other samples were taken at 6, 12, 18 and 24 hours after induction.

b. Interferon activity. The five samples were centrifuged and supernatants assayed for interferon activity (as shown in Table II). As can be seen from the table, the titer reached 5,250 units of interferon per 1 X 10^6 cells after six hours. The highest level of interferon activity, 10,000 to 10,250, units was reached between 18 and 24 hours after induction.

c. Isolation of RNA. Culture samples were taken at 0, 6, 12, 18 and 24 hours after induction of the cells and the cell RNA was then isolated. The amount of RNA extracted from each culture of induced cells is given in Table III.

TABLE II

KINETICS OF IFN-α SYNTHESIS
IN NAMALVA CELLS

Hours after induction	Interferon (Units per 1 x 10^6 cells)
0	275
6	5,250
12	5,500
18	10,000
24	10,250

The Poly(A)$^+$ mRNA was isolated from each RNA sample by chromatography on an oligo(dt) column as described in Methods.

To evaluate the characteristics of the RNA, some of the RNA samples were resolved by electrophoresis on one percent (w/v) agarose in Peacock's buffer (Appendix, #5a). The RNAs from cells induced 12 hours previously were resolved by electrophoresis on agarose gel, stained, and photographed. The characteristic bands of ribosomal RNA are seen in Figure 10. The results indicated that the prepared RNA was suitable for hybridization.

d. Dot blot hybridization. In order to measure the relative amounts of α-interferon (IFN-α) mRNA from induced Namalva cells at different times and to compare these with

TABLE III

TOTAL RNA RECOVERED FROM
INDUCED NAMALVA CELLS

Hours after induction	Total RNA (μg) recovered from cells 1 X 10^6)
0	5.29
6	6.33
12	5.62
18	6.21
24	6.00

the leukocyte mRNA controls, the following experiment, shown in Figure 11, was carried out. A 1.5 μg aliquot of poly(A)$^+$ mRNA was removed from each sample after 0, 6, 12, 18 and 24 hours of induction. After treatment with formaldehyde as described in Methods, three aliquots (shown in Columns 1, 2 and 3 of Figure 11) of each sample were spotted onto nitrocellulose paper using a dot blot apparatus. The first series of samples for each time point, A_1, B_1, C_1, D_1 and E_1, contained 0.75 μg of poly (A)$^+$mRNA.

The second series of samples A2, B2, C2, D2, and E2 contained 0.45 μg of poly (A)$^+$mRNA. The third series of samples, A3 and B3 contained 0.22 μg and C3, D3, E3, contained 0.15 μg of Poly(A)$^+$mRNA. In addition to Namalva poly(A)$^+$ mRNA, five different human leukocyte poly(A)$^+$ mRNAs were also spotted onto the same paper as

Fig. 10--Electrophoresis of RNA from induced Namalva cells on one percent (w/v) agarose gel in Peacock's buffer. The amount placed on lane A was 1.6 μg and in lane B, 0.8 μg of RNA obtained from cells 12 hours after induction. The 5S, 18S and 28S bands are indicated by arrows.

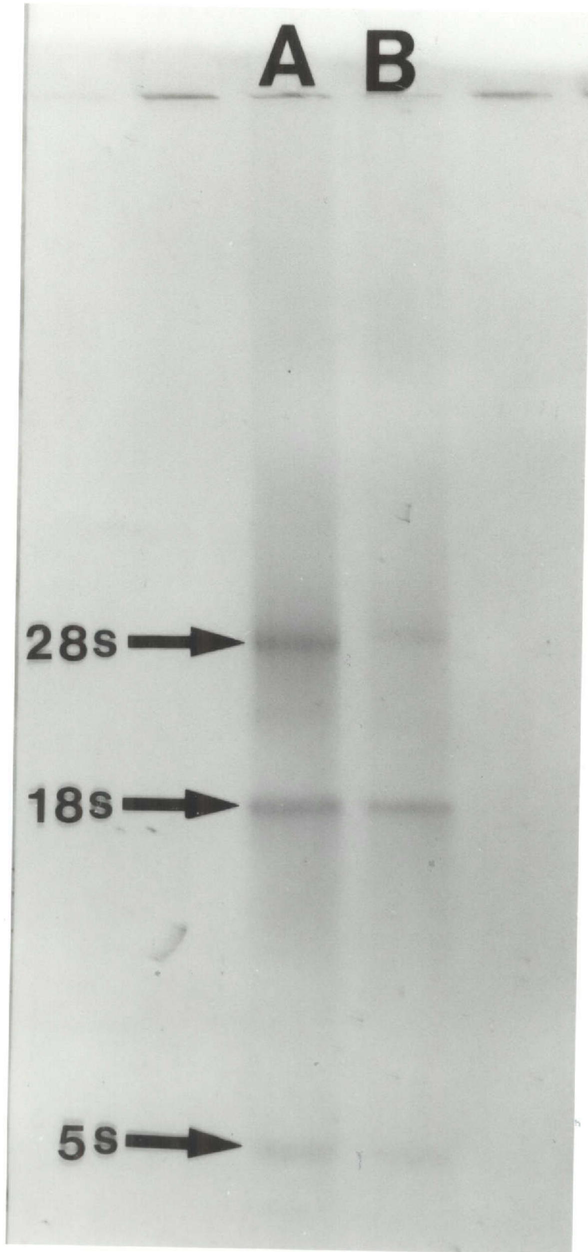
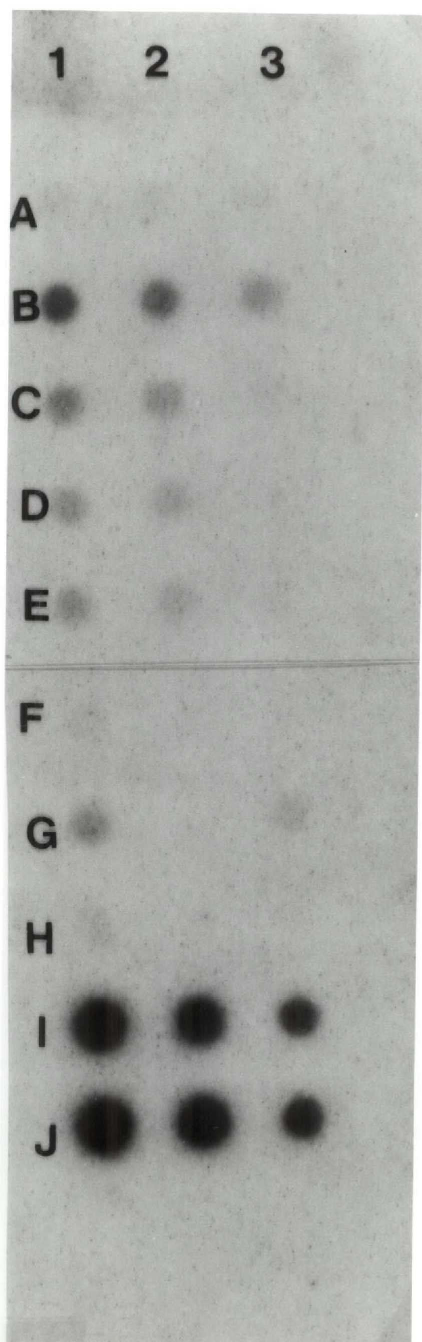

A B
28s
18s
5s

Fig. 11--Dot blot hybridization of induced Namalva cell poly(A)$^+$ mRNA and induced and uninduced leukocyte poly(A)+ mRNA with nick-translated M13mp11-IFN-αWA. Poly(A)$^+$ mRNA samples of Namalva cells and leukocytes were spotted onto nitrocellulose paper, hybridized with nick-translated IFN probe and autoradiographed. The autoradiogram shown was obtained after three days of exposure. The material in lanes A_1, B_1, C_1, D_1 and E_1 contained 0.75 µg of Poly(A)$^+$mRNA, lanes A_2, B_2, C_2, D_2 and E_2 contained 0.45 µg of Poly(A)$^+$mRNA, lanes A_3 and B_3 contained 0.22 µg of Poly(A)$^+$mRNA, lanes C_3, D_3 and E_3 contained 0.15 µg of Poly (A)$^+$mRNA of induced Namalva cells. The materials in lanes F_1 and H_1 contained 0.5 µg of Poly(A)$^+$mRNA, lanes F_2 and H_2 contained 0.3 µg of Poly(A)$^+$mRNA, lanes F_3 and H_3 contained 0.15 µg of Poly(A)$^+$mRNA of uninduced leukocytes. Lanes G_1, I_1, and J_1 contained 0.5 µg of Poly(A)$^+$mRNA, lanes G_2, I_2, and J_2 contained 0.3 µg and lanes G_3, I_3 and J_3 contained 0.15 µg, 0.12 µg and 0.12 µg of Poly(A)$^+$mRNA of induced leukocytes, respectively.


1
2
3
A
B
C
D
E
F
G
H
I
J

controls. Two samples, F and H were poly(A)$^{+}$ mRNA from uninduced cells and the other three (G, I and J) were poly(A)$^{+}$ mRNA from induced cells. After hybridization, all samples were autoradiographed and the film was developed after three days. The results shown in Figure 11 indicate that the level of α-interferon (IFN-α) message reached the maximum six hours after induction and then declined. The two poly(A)$^{+}$ mRNA samples isolated from uninduced leukocytes did not have the IFN-α message and therefore did not hybridize with the probe. The poly(A)$^{+}$ mRNA isolated from induced leukocytes G, I and J, however, hybridized with the same probe.

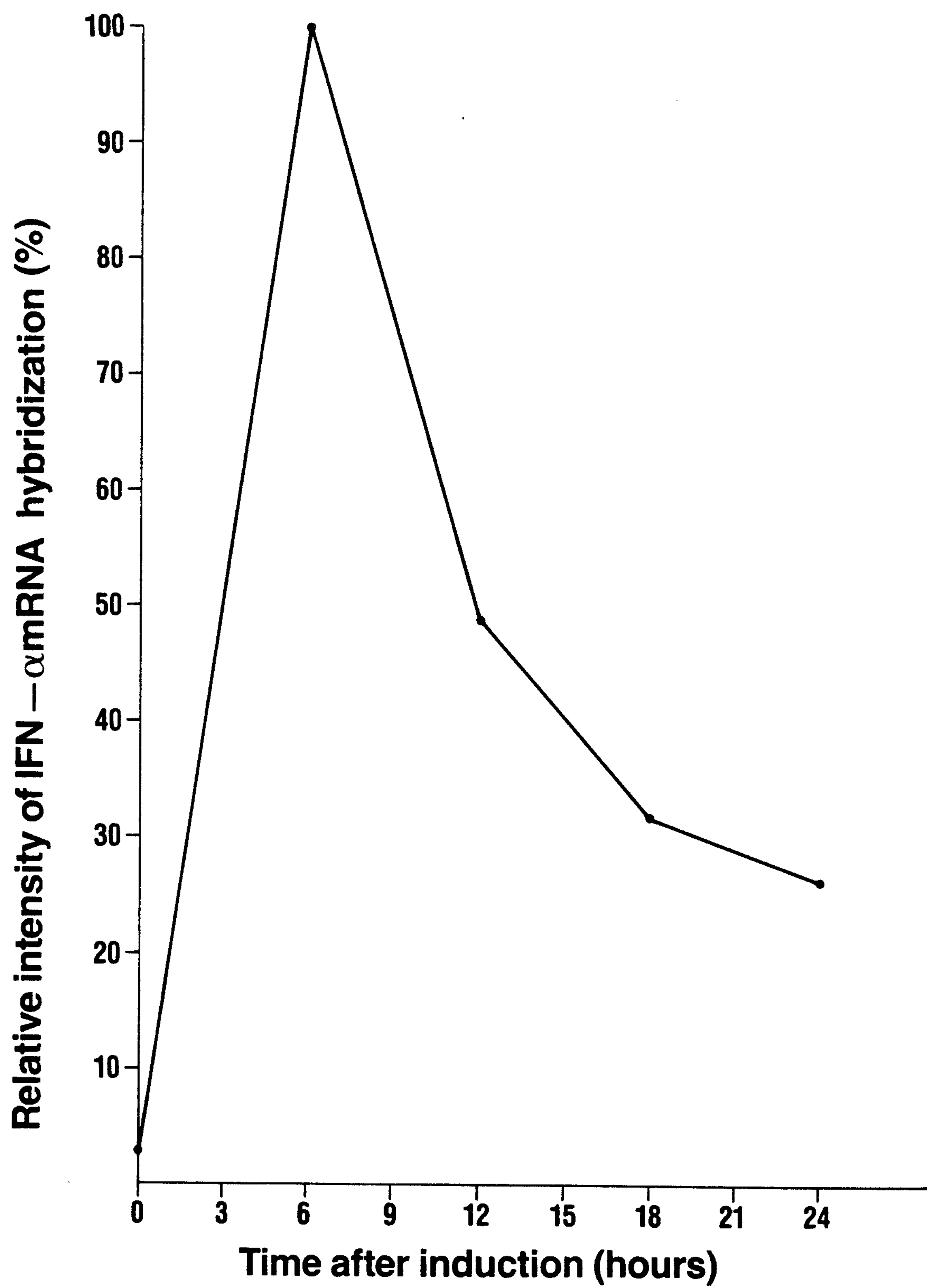
Quantitative Analysis of the Level of IFN-α mRNA

The poly (A)$^{+}$mRNA dots in Figure 11 were scanned to determine the amount of IFNmRNA. Results (Figure 12) indicate that a peak of IFN-α message occurred six hours after induction, followed by a slow decline in the level of IFN-α message. Whereas the maximal level of α-interferon mRNA appeared after six hours of induction (Figure 12), the maximal level of interferon protein appeared only after 18 hours of induction (Table II).

Dot Blot Hybridization of Namalva Cell Poly(A)$^{+}$ mRNA with Nick-translated M13mp11-IFN-α WA (Second Experiment)

a. Culture preparation. A total of 12 X 10^{9} cells were used for this experiment. Eight samples were taken

Fig. 12--Level of IFN-α mRNA in induced Namalva cells. The poly(A)$^+$ mRNA dots shown in Column 1 (A1 to E1) Figure 11 were scanned, the area of paper under each peak was cut, the paper weighed, and the percentage of its weight (relative to the greatest weight) was plotted against time after induction.



100
90
80
70
60
50
40
30
20
10
Relative intensity of IFN—αmRNA hybridization (%)
0
3
6
9
12
15
18
21
24
Time after induction (hours)

for RNA isolation. The first sample (zero hour) was taken immediately after induction and other samples were taken at 5, 6, 7, 8, 9, 10 and 12 hours after induction.

b. Interferon activity. All samples taken for interferon assay showed induction. At 24 hours, the level of interferon activity reached 14,500 units per 1×10^6 cells.

c. Isolation of RNA. Approximately 1.5×10^9 cells were harvested at each time of the eight time intervals. Total cell RNA and poly$(A)^+$ mRNA from each sample were isolated as described in Methods. The amount of RNA, extracted per 1×10^6 of cells is given in Table IV.

d. Dot blot hybridization. In order to measure the relative amounts of IFN-α mRNA from induced Namalva cells at different times after induction, the following experiment, shown in Figure 13, was carried out. A 1.5 μg aliquot of poly$(A)^+$ mRNA of each sample (0, 5, 6, 7, 8, 9, 10 and 12 hours) was treated as described in the interferon dot blot experiment. Three aliquots of each poly$(A)^+$ mRNA were spotted onto nitrocellulose paper using a dot blot apparatus. The first series of samples, A_1, B_1, C_1, D_1, E_1, F_1, G_1 and H_1 contained 0.9 μg of poly$(A)^+$ mRNA. The second series of samples, A_2, B_2, C_2, D_2, E_2, F_2, G_2, H_2 contained 0.3 μg of poly$(A)^+$ mRNA, and the third series of samples,

TABLE IV

TOTAL RNA RECOVERED FROM INDUCED NAMALVA CELLS

Hours after induction	Total RNA (µg) recovered from 1 X 10^6 cells
0	8.60
5	6.93
6	5.47
7	8.27
8	6.87
9	8.87
10	7.93
12	5.67

A_3, B_3, C_3, D_3, E_3, F_3, G_3 and H_3 contained 0.1 µg of poly(A)$^+$mRNA. After spotting RNAs onto nitrocellulose paper and hybridization, the samples were autoradiographed (Figure 13). In confirmation of the results found in the first experiment, the level of IFN-α mRNA reached the maximum at six hours after induction and then declined.

Quantitative Analysis of the Level of IFN-α mRNA

The poly(A)$^+$mRNA dots, indicated in column 1 in Figure 13 were scanned to measure the amount of IFN mRNAs at different time intervals (Figure 14). As can be seen in Figure 14,

Fig. 13--Dot blot hybridization of induced Namalva cell poly(A)$^+$ mRNA with nick-translated M13mp11-IFN-αWA. The material in Rows A through H is poly(A)$^+$ mRNA from induced cells after 0, 5, 6, 7, 8, 9, 10 and 12 hours of induction. Column 1 0.9 μg, column 2 0.3 μg, and column 3, 0.1 μg of poly(A)$^+$ mRNA from induced Namalva cells. The film was developed after 19 hours of exposure.

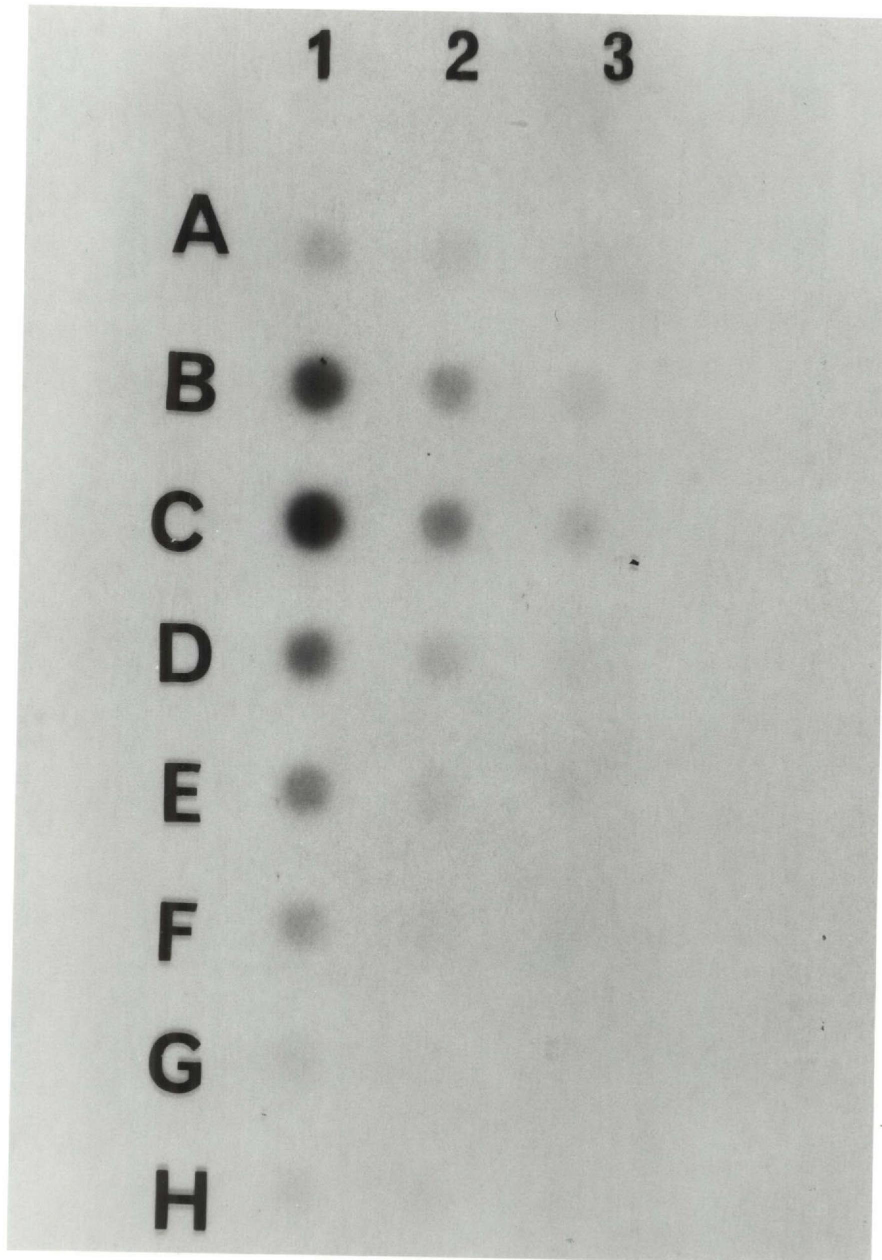
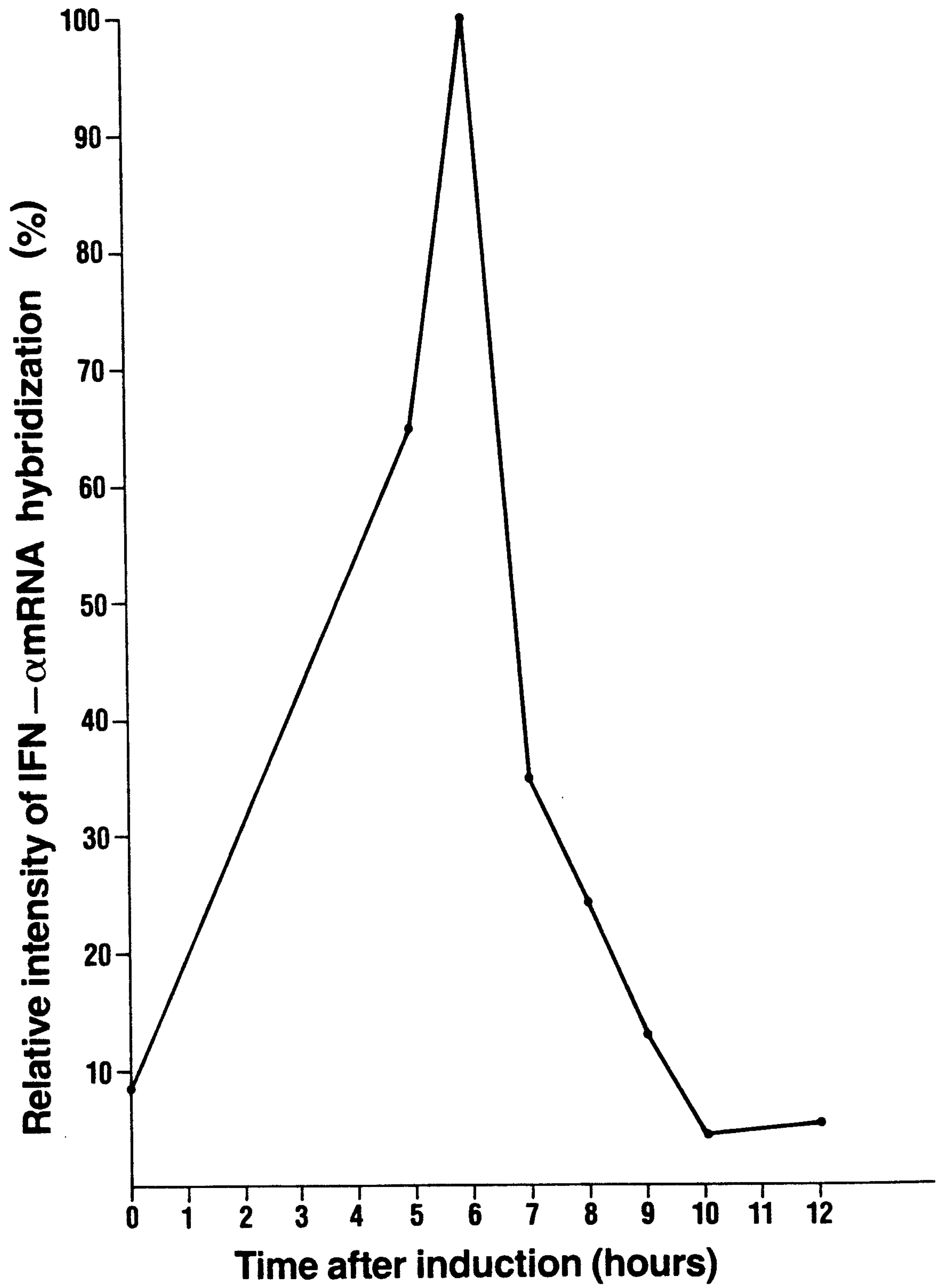

1
2
3
A
B
C
D
E
F
G
H

Fig. 14--Level of IFN-α mRNA in induced Namalva cells. The poly(A)$^{+}$ mRNA dots shown in column 1 (A1 to H1) Figure 13 were scanned, the area of paper under each peak was cut, the paper weighed and the percentage of its weight (relative to the greatest weight) was plotted against time after induction.



Relative intensity of IFN —αmRNA hybridization (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
Time after induction (hours)

there was a peak of IFN-α mRNA six hours after which the level declined.

Northern Hybridization of Namalva Cell RNA with 18S Ribosomal DNA and with IFN-α WA Gene

The RNA used in this experiment was from the previous interferon experiment. The RNA from the six hour sample and poly(A)$^+$ mRNA from the zero and six hour samples were used. The RNA samples, either denatured with glyoxal or in natural form, were resolved by electrophoresis on 1.5 percent (W/V) agarose in sodium phosphate buffer. In order to assess the integrity of the RNA isolated from induced Namalva cells, one lane containing the RNA was removed from the nine-lane gel [(as described in Figure 16) now containing only eight lanes] and stained with methylene blue. This is shown in Figure 15. Because the results from Figure 15 indicated that the RNA was intact, the Northern transfer (Figure 16) was carried out. The rest of the samples, in the other eight lanes, Figure 16 were transferred onto nitrocellulose paper. After overnight transfer, the paper was cut between lanes D and E, heated and saved for hybridization. One piece of nitrocellulose paper (on left in figure 16) contained RNA samples and the other piece contained poly(A)$^+$ mRNA samples. The RNA samples were hybridized with nick-translated pDF8, a subclone of 18S rat ribosomal DNA in pBR322. The Poly(A)$^+$

Fig. 15--Electrophoresis of glyoxalated RNA from Namalva cells. Total cell RNA (excised lane from gel described in Figure 16) (9.72 μg) was glyoxalated and resolved by electrophoresis on a 1.5 percent (w/v) agarose gel in sodium phosphate buffer overnight. The gel was stained in 0.2 percent (w/v) methylene blue and destained in water. The 18S and 28S bands are indicated by arrows.

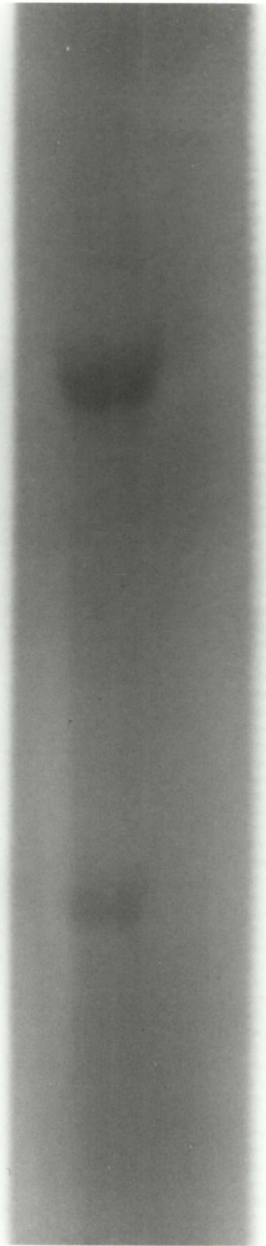

28S
18S

Fig. 16--Northern hybridization of Namalva cell RNA with a ribosomal probe, nick-translated pDF8, and Namalva cell poly(A)$^+$ mRNA with an IFN probe, nick-translated M13mp11-IFN-αWA. The material in lanes A and H contained end labeled λ DNA digested with E coRl and Hind III, lanes B and C contained 32.4 and 226.8 ng of denatured RNA, respectively. Material in lane D contained 3.24 μg of native RNA, and lane F contained 10 μg of denatured Poly(A)$^+$mRNA, isolated from uninduced cells at zero hour. Material in lane G contained denatured poly(A)$^+$mRNA isolated from uninduced cells six hours after induction. Lanes E contained no RNA. The film was developed after 16 hours of exposure. Kb deontes kilobase.

A B C D E F G H

kb

21·2

5·1
4·9
4·2
3·5

2·0
18s → 1·9
1·5
1·3

0·94
0·83

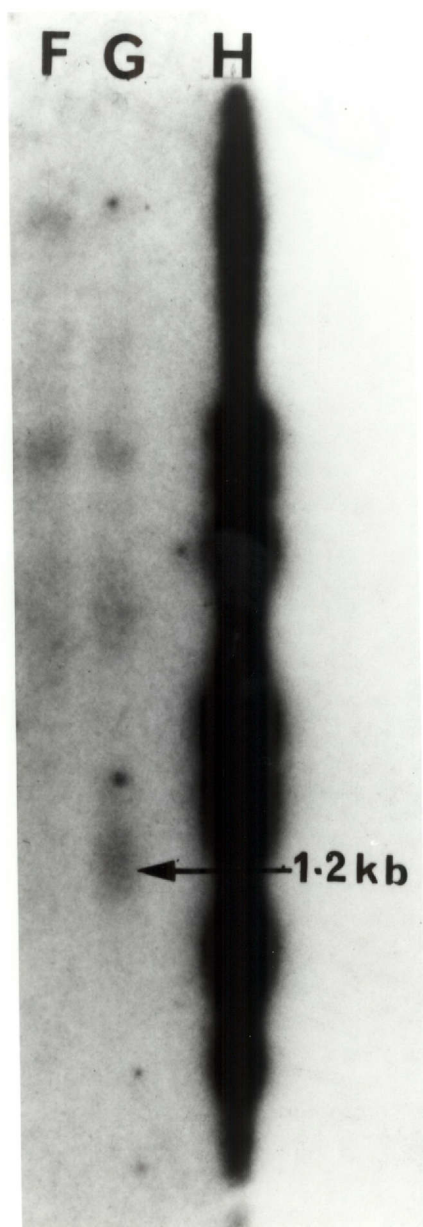
0·56

mRNA samples were hybridized with nick-translated M13mp11-IFN-α WA. These results are shown in Figure 16. The following remarks are made regarding Figure 16. The 18S band is shown in lanes A, C, and D. The native RNA sample (lane D) however, did not migrate at the same speed as the denatured samples (lanes A and C) during electrophoresis. After the autoradiograms were exposed for 16 hours, interferon mRNA was not detected. Accordingly, Poly $(A)^{+}$mRNA in lanes F, G and H was exposed for a longer time, i.e. six days. After the second exposure, The α-interferon (IFN-α) mRNA was detected and found to have an approximate size of 1.2 kb (Figure 17). This indicated that the IFN-α mRNA had been induced.

Specificity of c-*myc* Probe

It is known that human lymphoblastoid cells contain oncogenes such as the c-*myc* oncogene of Daudi cells. This human lymphoblastoid cell line, like the Namalva line, also originated from a Burkitt's lymphoma but Daudi cells produce much lower levels of interferon than Namalva cells after in-duction with Sendai virus. Therefore, it is deemed of impor-tance to determine whether the c-*myc* oncogene is expressed in Namalva cells and if so to what degree compared to Daudi cells. The importance lies in the fact that the greater production of interferon makes Namalva the cells of choice but if the oncogene is active then their use in cancer therapy would not be feasible.

Fig. 17 -- Northern hybridization of Namalva cell poly(A)$^+$ mRNA with a nick-translated M13mp11-IFN-α WA (long exposure of lanes F and G of Fig. 16). The 1.2 kb IFN mRNA is indicated. The film was developed after 6 days exposure. Lane H contains labeled λ Hind III DNA marker.


F
G
H
1·2 kb

It was deemed necessary to prove the specificity of the c-myc probe. To accomplish this, five cell lines were selected; Namalva and Daudi cells from human Burkitt lymphomas and PLC/PRF/5 cells from a human hepatoma. The other two were BALB/c 3T3 and H35, a mouse fibroblast cell line and a rat hepatoma cell line respectively. The mRNA of each cell line was hybridized with nick-translated pHSR-1 using the c-myc probe and the results are shown in Figure 18. No hybridization was detected for the BALB/c 3T3 and H35 mRNAs but the Namalva, Daudi and PLC/PRF/5 mRNAs showed considerable hybridization. This indicates that the c-myc probe was specific for human cells and that it did not hybridize with the two rodent cell lines tested.

Northern Hybridization of RNA Isolated from Namalva Cells, Daudi Cells and Leukocytes with c-myc Probe

In order to determine whether or not the RNA isolated from Namalva cells, Daudi cells and leukocytes selected were suitable for Northern hybridization 5 to 8 µg of the RNAs were glyoxalated and resolved by electrophoresis. The characteristic 18S and 28S rRNA bands were observed with no apparent degradation (Figure 19), indicating that the RNA was suitable for hybridization experiments.

In order to investigate that the Namalva cells, Daudi cells and leukocyte contained c-myc oncogene, Northern hybridization was performed. For Northern hybridization, 40 µg of each RNA from Namalva cells, Daudi cells and leukocytes

Fig. 18--Quick blot hybridization of mRNA from Namalva, Daudi, PLC/PRF/5, BALB/c 3T3, and H35 cells with c-*myc* probe. The material in A_1, A_2 and A_3 contained mRNA from 1 X 10^6, 0.5 X 10^6, and 0.25 x 10^6 Namalva cells, respectively. The material in B_1, B_2 and B_3 contained mRNA from 1 x 10^6, 0.5 x 10^6 and 0.25 x 10^6 Daudi cells, respectively. The material in C_1, C_2 and C_3 contained mRNA from 0.5 x 10^6, 0.25 x 10^6 and 0.125 x 10^6 BALB/c 3T3, respectively. The material in D_1, D_2 and D_3 contained mRNA from 0.5 x 10^6, 0.25 x 10^6 and 0.125 X 10^6 PLC/PRF/5 cells, respectively. The material in E_1, E_2 and E_3 contained 0.5 x 10^6, 0.25 x 10^6 and 0.125 x 10^6 H35 cells, respectively. The autoradiogram was obtained after a 17-hour exposure.

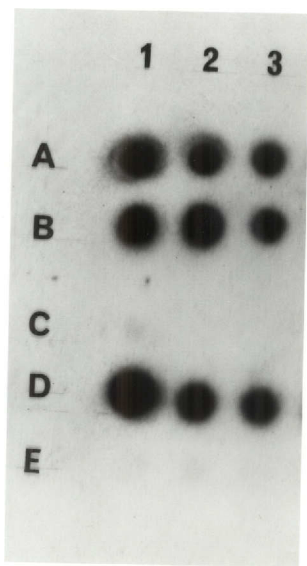
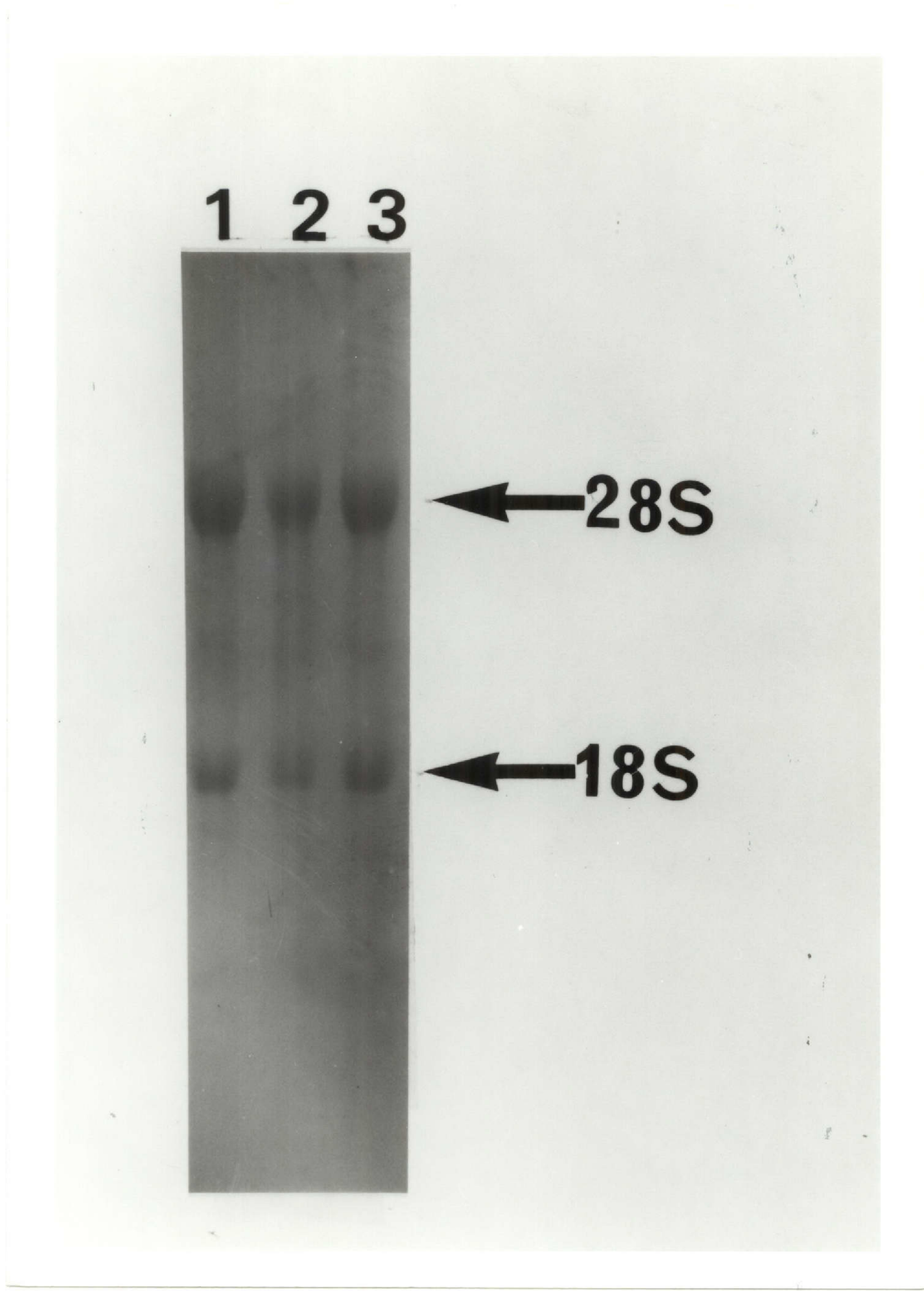

1
2
3
A
B
C
D
E

Fig. 19--Electrophoresis of glyoxalated RNA from Namalva and Daudi cells and leukocytes. Typically 5 to 8 μg of each RNA sample were glyoxalated, resolved by electrophoresis on a 1.5 percent (w/v) agarose gel in phosphate buffer and stained in 0.2 percent (w/v) methylene blue as described in Methods. The material in lanes 1, 2 and 3 contained total cell RNA from Namalva cells, leukocytes and Daudi cells, respectively.



1 2 3
28S
18S

together with an λ HindIII DNA marker were glyoxalated and resolved by electrophoresis on 1.5 percent (w/v) agarose in sodium phosphate buffer and further processed for hybridization as described in Methods.

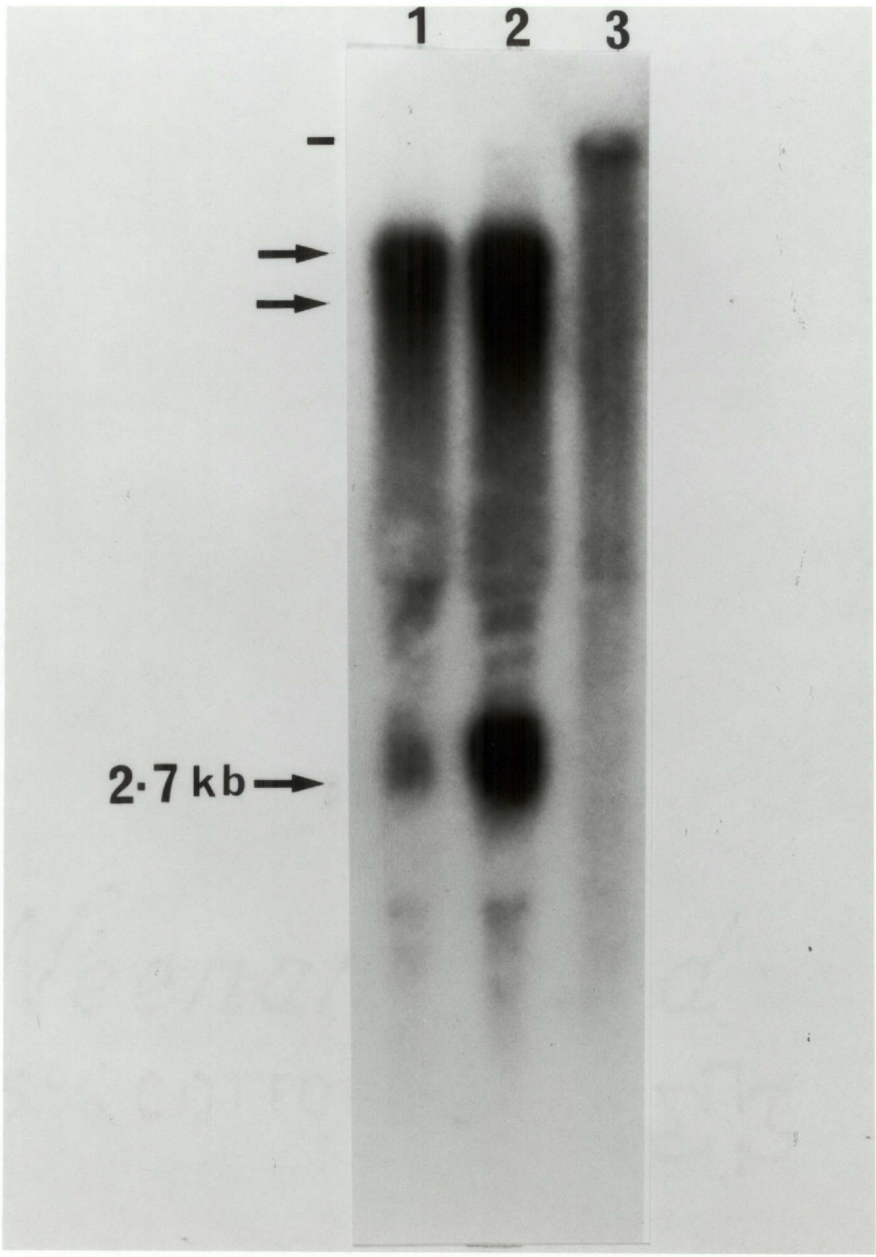
Hybridization of Namalva and Daudi cell RNAs with c-myc probe revealed a transcript with an approximate size of 2.7 kb. By comparing the intensity of radioactivity in autoradiograms of Namalva and Daudi RNAs (Figure 20), the extent of hybridization of Namalva cells was found to be only one-tenth that of the RNA of Daudi cells. Hybridization of leukocyte RNA was not detected even after 30 hours of autoradiography exposure.

In addition to the 2.7 kb transcript, hybridization of Namalva and Daudi RNA with c-myc probe revealed two other transcripts larger than 23 kb in Namalva and in Daudi RNA. Similar transcripts however, were not observed in leukocyte RNA (Figure 20). It is noteworthy that the lack of hybridization of leukocyte RNA with the c-myc probe did not appear to be due to leukocyte RNA degradation (Figure 19).

Northern Hybridization of RNA Isolated from Namalva Cells, Daudi Cells and Leukocytes with c-Ha-ras Probe

It has been shown that c-myc and c-Ha-ras oncogenes act synergistically in tumorigenesis (70, 130). Therefore, Northern hybridization was carried out to see whether c-Ha-ras oncogene is also expressed in Namalva cells.

Fig. 20--Northern hybridization of RNA isolated from Namalva cells, Daudi cells and leukocytes with c-myc probe. Each RNA sample (40 µg) was glyoxalated and resolved by electrophoresis on a 1.5 percent (w/v) agarose gel in sodium phosphate buffer. The samples were transferred to nitrocellulose paper and hybridized with c-myc probe as described. After washing, autoradiography was performed for 30 hours. The material in lane 1, 2 and 3 contained RNA from Namalva cells, Daudi cells and leukocytes respectively. A Lambda λ DNA digested with HindIII was used as a size marker. The arrows (→) indicate the large molecular size bands seen in Namalva and Daudi RNA. The dash (—) denotes the origin of the gel. The 2.7 kb band is also indicated by arrow.


1
2
3
2·7 kb

After autoradiography of the Northern hybridization of RNA samples with c-*myc* probe, filters were heated in boiling water to remove the c-*myc* probe (115). The samples were then rehybridized, this time with nick-translated pT24-C3.

After six hours of autoradiography exposure, a major transcript of about 6.1 kb appeared in Namalva and Daudi cell RNA indicating that c-Ha-*ras* was expressed in Namalva and Daudi cells (Figure 21). The appearance of a faint hybridization transcript in leukocyte RNA could have been due to base sequence homology of the RNA with the c-Ha-*ras* probe since the leukocytes are nontransformed cells and do not contain oncogenes. A longer exposure (Figure 22) revealed another transcript with an approximate size of 1.8 kb in Namalva and Daudi cell RNA, but not in leukocyte RNA.

Fig. 21--Northern hybridization of RNA isolated from Namalva cells, Daudi cells and leukocytes with c-Ha-*ras* probe (short exposure). The filter used in the c-*myc* Northern hybridization, shown in Fig. 20, was placed in boiling water for ten minutes and the RNA was rehybridized with nick-translated c-Ha-*ras* probe. The material in lanes 1, 2, and 3 contained RNA from Namalva cells, Daudi cells and leukocytes respectively. The 6.1 Kb band is indicated by an arrow.

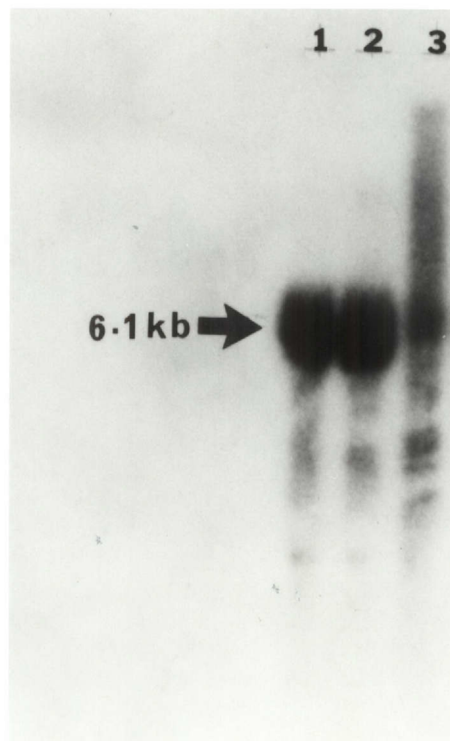
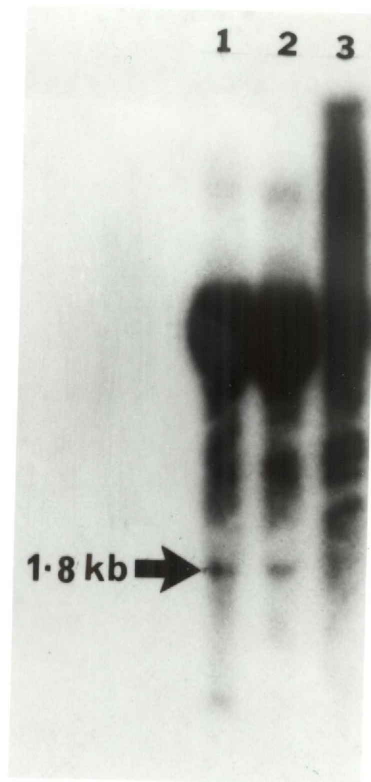

1 2 3
6·1kb

Fig. 22--Northern hybridization of RNA isolated from Namalva cells, Daudi cells and leukocytes with c-Ha-*ras* probe (long exposure). The filter used in Fig. 21 was re-exposed for 17 hours. The 1.8 kb band is indicated by an arrow.


1
2
3
1·8 kb

CHAPTER IV

DISCUSSION

The Northern blot method of hybridization with a synthetic oligomer probe confirmed the specificity of the probe as well as the integrity of the RNA. Also, it was possible to compare the degree of hybridization in dot and Northern blots. It was found that the degree of hybridization obtained after Northern transfer of an RNA sample was one-tenth that obtained from an equivalent RNA sample applied directly to the nitrocellulose paper (Figure 6). This means that the dot blot method was more suitable for measuring the level of IFN-α mRNA than the Northern blot. It is pertinent to note that these hybridizations were not only adequate for testing the integrity of RNA preparations, but could also be used diagnostically for distinguishing certain genetic disorders, i.e. sickle cell anemia in which there is only a one-base difference between the mRNA of normal cells and that of the diseased cells. For detecting such a genetic disease, two oligomers are made, one complementary to the sequence of the normal gene and one complementary to the sequence of the abnormal gene. In this way, such a hybridization using the synthetic oligomers can distinguish between normal and abnormal genes (4).

It was essential in pursuit of the goals of this project to determine whether the interferon mRNA could be induced and to determine the quantitative differences between the interferon (IFN) mRNA in induced and uninduced Namalva cell poly(A)+ mRNA. It was also necessary to determine whether Namalva cell RNA could hybridize with the interferon probe. Since in uninduced cells, the interferon mRNA is not produced, it cannot be detected by hybridization with the interferon probe. Dot blot hybridizations showed no affinity between induced and uninduced cells with respect to total cell RNA when the interferon probe was used (Figure 9). The absence of hybridization with total cell RNA may have been due to interference of ribosomal RNA with the probe. However, both induced and uninduced cells had poly$(A)^+$mRNA hybridized with the interferon probe.

While the amount of poly$(A)^+$mRNA from uninduced cells applied onto the nitrocellulose paper was almost twice that of poly$(A)^+$mRNA from induced cells, it hybridized much less than the induced Namalva cell poly$(A)^+$ mRNA (Figure 9). Thus as expected, the poly$(A)^+$ mRNA from uninduced cells hybridized less than the induced due to the presence of a low level of interferon mRNA; the poly(A)+ mRNA from induced cells, on the other hand, despite the smaller quantity used, hybridized more than did the uninduced cells.

One reason for performing the interferon assays was to insure that the cells had been induced to produce interferon. Another reason was to compare the level of α-interferon (IFN-α) with the level of IFN-α mRNA produced during a 24 hour period to show the relationship between level of IFN-α mRNA and the interferon protein synthesized. Results of the interferon assay indicated a maximal content of some 10,000 units of interferon per 1 X 10^6 cells at 18 to 24 hours. Six hours after addition of Sendai virus, the level of interferon had reached 5,250 units per 1 X 10^6 cells, almost the same as at 12 hours 5,500 units per 1 X 10^6 cells. The level of interferon content reached a plateau at approximately 18 hours. The level of interferon at zero hours was about 275 units per 1 X 10^6 cells; this was higher than expected and may have resulted from the interference of the Sendai virus with the assay. In the first study of interferon production by lymphoblastoid cells, Strander _et al_. (107) obtained 1,100 units per 1 X 10^6 cells 24 hours after induction of Namalva cells with Sendai virus. Zoon _et al_. (132), also using Sendai virus for induction of interferon, reported titers of about 8,000 units per ml 20 hours after induction.

The production of IFN-α mRNA was found to be maximal at six hours after induction and declined slowly thereafter (cf. Figure 13). Induction of IFN-α mRNA was also determined in human leukocytes using similar methods.

A comparison of the level of interferon activity (Table V) and the level of IFN-α mRNA (Figure 12) in the experiment using the dot blot assay revealed a significant difference between the two. However, at 18 to 24 hours, interferon levels had increased to the maximum while the IFN-α mRNA level had declined.

To date, several investigators have reported the kinetics of induction of IFN-α mRNA in Namalva cells (9, 26, 49, 84, 90, 99, 100). Shuttleworth et al. (90) found maximum levels of IFN-α mRNA production after nine hours of incubation of butyrate-treated Namalva cells; the level of IFN-α reached its maximum at approximately 12 hours after induction. The same laboratory obtained similar results using the oocyte injection method (84).

The results of these experiments namely, maximum level of IFN-α mRNA six hours after induction, are in accord with those of Colman and Morser (26) who observed that the amount of IFN-α (determined by assay of interferon extracted from injected oocytes) reached a maximum within six hours of induction. The kinetics of IFN-α synthesis was not reported by these workers.

Raj et al. (90) used a shorter priming time, less than two hours and less virus for induction than were used in these experiments. Using the cytoplasmic dot blot hybridization technique, they found both α- and β - IFN mRNAs produced at 12 to 15 hours after induction. The level of α -

and β - interferon reached the maximum at 24 hours after induction. The autoradiogram or the scan of the autoradiogram from which results were obtained was not shown (90). Rather, Raj et al. (90) showed only an overexposed autoradiogram of dots from which it was difficult to reach conclusions. The time intervals chosen for measurement of the IFN-α mRNA levels were 3, 6, 12, 15, and 24 hours after induction (90). It was observed that the maximum level of IFN-α mRNA occured six hours after induction. The results obtained in this study are not in agreement with the results of Raj et al. (90) who found that the level of IFN-α mRNA reached its maximum at 12 to 15 hours. The differences between the results of Raj et al. (90) and these might be due to: (a) differences in the induction system since they used a shorter priming time and more virus (100 hemagglutinating units of Sendai virus per 1 X 10^6 cells) while a longer priming time and less virus (37.5 hemagglutinating units of Sendai virus per 1 X 10^6 cells) were used in this study; (b) they used the dot blot hybridization using cytoplasmic RNA (129), while poly(A)$^+$ mRNA dot blot hybridization in which the poly(A)$^+$ mRNA isolated from total cell RNA was used in these studies; or (c) the time intervals chosen for sampling during the assay.

Berger et al. (9), using the oocyte assay, reported a broad peak of IFN-α mRNA level beginning eight hours after induction with Newcastle disease virus. The maximum

level, five units per ten oocytes, was maintained six hours and decreased at 17 hours after induction. The level of IFN-α was constant between 11 and 14 hours after induction. The interferon concentration reached a plateu at 14-hours, 5,010 units per milliliter of culture. Hiscott *et al*. (49), using S1 mapping, developed a technique for quantitation of the different species of interferon. These workers found that the highest copy number of IFN-α , 940 per cell, occurred four hours after induction. No transcripts of the *alpha* interferon message were detected by Hiscott *et al*. (49) in samples taken 22 hours after induction. The maximum copy number in these samles was 940 per cell and these were seen four hours after induction. Ten hours after induction, the copy number was 850.

This investigation is unique since a subcloned Namalva cell line was employed together with a newly cloned interferon gene. In our work, the gene was used as a hybridization probe (IFN-α WA). The original Namalva cells (86) produce 80 to 90 percent of IFN-α and 10-20 percent of IFN-β after induction (7, 33) whereas the subcloned cells produce 80 to 100 percent of IFN-α . The newly cloned IFN-α gene (116) had not been previously used in studies of IFN-α mRNA. Because of the unique base sequence of this gene, it might be used to hybridize with certain other IFN-α genes. On the other hand, the fact that the level of IFN-α mRNA species varied in induced Namalva cells (49), might

contribute to the differences in the results of this and other studies of IFN-α mRNA level in which different IFN-α probes were used.

The dot blot hybridization technique using poly(A)$^+$ mRNA was employed to quantitate the level of IFN-α mRNA. In this study, dot blot hybridization was used for the study of IFN-α mRNA level in induced Namalva cells. The level of IFN-α mRNA can also be measured by an oocyte RNA translation assay. However, the advantage of the former transcription assay is that one can measure the IFN-α mRNAs which are synthesized but might or might not be translated.

Conditions of induction differed from those previously used by others in this type of work. For example, human leukocyte interferon was used for priming Namalva cells for two hour periods before inducing them with 37.5 hemagglutinating units of Sendai virus per 1 X 10^6 cells. Raj et al. (90) used Namalva interferon for priming of Namalva cells for one hour and used 100 hemagglutinating units of Sendai virus per 1 X 10^6 cells for induction. Hiscott et al. (49) used 100 to 1,000 hemagglutinating units of Sendai virus per 1 to 15 X 10^6 cells for induction of Namalva cells. Thus, each factor in induction of Namalva cells, such as the type of inducer, primer, and period of priming and also the treatment of cells with chemicals such as butyrate (99) might affect the production of IFN-α and IFN-α mRNA.

Hybridization of Namalva cell RNA with ribosomal probe was carried out for two reasons (Figure 16). First, hybridization was conducted to evaluate the condition of RNA hybridization employed and the temperature at which the RNA was hybridized. It was important to determine if RNA could be denatured by the conditions employed and then could hybridize with the ribosomal probe. The hybridization of RNA with the interferon probe was conducted under the same conditions as the hybridization with the ribosomal probe. In this way the hybridization of RNA with the ribosomal probe served as a control for the hybridization with the interferon probe. The second reason for hybridization was to have a labeled RNA marker which makes it easier to judge the size of the sample when compared with an unlabeled marker.

Northern hybridization of poly(A)$^+$ mRNA with the IFN-α probe (Figures 16 and 17) allowed calculation of the size of the IFN-α mRNA. The first purpose of using poly(A)$^+$ mRNA from the zero hour sample in the hybridization was to compare it with poly(A)$^+$ mRNA from induced cells and to determine if the virus had induced the production of IFN-α mRNA since the uninduced poly(A)$^+$ mRNA should not hybridize with the probe. The other purpose of using poly(A)$^+$ mRNA from the zero hour sample, was to observe the specificity of the interferon probe used in the hybridization experiment since nonspecific probe might hybridize in an indescriminate

way with induced and uninduced poly(A)$^+$ mRNA. Therefore, the poly(A)$^+$ mRNA isolated from the zero hour sample served as a suitable negative control.

Hybridization of poly(A)$^+$ mRNA from the six-hour sample with the IFN-αWA probe (Figures 16 and 17) revealed an IFN-α mRNA transcript with an approximate size of 1,200 bases. This is in the same size range as that reported for the major species of IFN-α mRNAs by other workers (90, 91, 95, 96, 100). Comparable size range is important since it indicates that the message used in this study was in the same size range as that of other IFN-α mRNA studied.

Results of the study reported here indicate that no hybridization was detected with the poly(A)$^+$ mRNA from the zero hour sample (Figure 17). The absence of the 1.2 kb band is compatible with the low level of IFN-α mRNA found in the zero hour sample. The 1.2 kb transcript appeared only in the poly(A)$^+$ mRNA from induced cells (Figure 17) at six hours, the maximal level of mRNA produced, indicating the induction of IFN-α mRNA.

Another aspect of this investigation concerns the expression of c-myc and c-Ha-ras oncogenes in Namalva cells. This phase of the study developed as a result of previous work on the expression of these genes in other lymphoblastoid cells (27 - 30, 34, 62, 98, 112, 128). The obvious hazard of using interferon produced from these cells in cancer treatment made it necessary to ascertain if

the Namalva cells expressed oncogene(s). These two oncogenes were selected for further study because of the synergism they produced in tumorigenesis. For example, rat embryo fibroblasts undergo transformation when co-transfected with active myc and Ha-ras oncogenes (70); however, transformation of rat embryo fibroblasts with either myc or ras alone do not lead to cell transformation (70).

The c-myc probe used in this study had a total size of 13.3 kb (including the vector). Therefore, a dot blot hybridization experiment was set up to insure that the probe was sufficiently specific. Subsequently the hybridization of the c-myc probe with mRNAs of five cell lines was tested. The results indicated that under the conditions of hybridization used, only human RNAs hybridized with the c-myc probe. Such results were predictable since the c-myc gene had been isolated from a human neuroendocrine tumor line, CoLo 320 HSR (2). The results confirmed the specificity of the c-myc probe since the nonspecific probe could hybridize in a non-discriminate way with any other gene which had some base homology with the probe.

Suitable controls were needed to investigate the expression of c-myc and c-Ha-ras; therefore, Daudi cells were selected as a positive control. The expression of both c-myc and c-Ha-ras in Daudi cells had been reported earlier (62, 128), and the Daudi cell line, like Namalva, is a lymphoblastoid cell line isolated from Burkitt's lymphoma

(65). Choosing a negative control was a difficult task since the negative control had to be "normal" and of human origin. In previous oncogene expression studies, human leukocytes were used as the negative control (45) but only the dot blot procedure was used. The use of leukocyte RNA in a Northern hybridization with the c-myc oncogene had not been reported, but the assumption was that leukocyte RNA might provide a suitable negative control for c-myc and c-Ha-ras oncogene expression in Namalva cells.

The major transcript, 2.7 kb (Figure 20), in Namalva cell RNA which hybridized with the c-myc probe was the same size as that found in Daudi cell RNA (128). The results of the experiments indicate that c-myc is expressed in Namalva cells, but at lower levels than those found in Daudi cells.

The two RNA transcripts larger than 23 kb, (Figure 20) which hybridized with c-myc in Namalva and Daudi cell RNA could be due to hybridization to an RNA species which has homology with c-myc or to the existence of a precursor of the c-myc RNA. Hybridization with these high molecular weight transcripts appeared to be significant due to their absence from human leukocyte RNA.

In this study, the Northern hybridization of leukocyte RNA with a c-myc probe revealed no hybridization. The absence of hybridization in leukocyte RNA did not appear to be due to RNA degradation (Figure 21), and tests established

that leukocyte RNA could serve as a suitable negative control for c-myc expression studies.

Expression of the c-Ha-ras oncogene has been reported in a rat liver tumor, a rat hepatoma cell line (80), a human hepatoma cell line (85), human lymphoblastoid cell lines (128), and several human tumors such as Ewing sarcoma, fibrous dysplasia, stomach carcinoma, and lymphosarcoma (111). Since there are no reports of c-Ha-ras expression in Namalva cells, and because of the synergism of this oncogene with c-myc, the expression of c-Ha-ras in Namalva cells was investigated. The Northern hybridization of Namalva cell RNA with the c-Ha-ras probe revealed a major transcript of approximately 6.1 kb (Figure 21). A faint transcript in the same size range also appeared in the leukocyte RNA. This might have been due to homology of leukocyte RNA with the c-Ha-ras probe. Autoradiography, employing a long time exposure, revealed a transcript with an approximate size of 1.8 kb (Figure 22) compared to the 6.1 kb transcript of Namalva cells. As was expected, this transcript was not seen in leukocyte RNA.

The 6.1 kb transcript observed in Namalva and Daudi RNAs was in the same size range as reported earlier (128). However, the sizes of c-Ha-ras mRNAs in different tumors and cell lines have been been reported to range from 1.2 to 6.5 kb (80, 85, 111, 128).

One pertinent observation in the c-Ha-ras hybridization experiment as seen in the 1.8 and 6.1 kb transcripts (Figures 21 and 22) indicates that the intensities of the Namalva cell RNA signals are almost as large as those in Daudi RNA. The intensity of the 2.7 kb transcript in the Namalva cell RNA in the c-myc hybridization experiment (Figure 22) was at least one-tenth that of Daudi cell RNA. Therefore, these results on c-myc and c-Ha-ras hybridization (Figures 20 - 22) indicate that the ratio of c-myc to c-Ha-ras expression is lower in Namalva cells than in Daudi cells.

The purpose of this study was to investigate the kinetics of IFN-α and IFN-α mRNA production in induced Namalva cells and to ascertain if these cells, which produce high levels of IFN-α after induction express any oncogene(s).

It was also important to investigate the expression of oncogenes in these cells since the interferon produced from these cells is used for treating cancer patients (39).

It was found that the level of IFN-α mRNA reached its maximum at six hours after induction and slowly declined thereafter. The level of IFN-α however reached its maximum at 18 hours after induction. Northern blot analysis, indicates that the size of IFN-α mRNA was 1.2 kb. Study of the kinetics of IFN-α mRNA will help to

understand more of the mechanism of interferon production *in vivo*. It was also shown that Namalva cells express c-*myc* and c-Ha-*ras* oncogenes. The degree of expression of c-*myc* and c-Ha-*ras* was different in Namalva cells when these were compared to Daudi cells. In this study, c-*myc* and c-Ha-*ras* were both expressed in Namalva cells. Information such as this is important because Namalva cell interferon is presently used for cancer therapy. Therefore these results should serve as a caveat in the clinical use of interferon and then only with the understanding that the c-*myc* and c-Ha *ras* are expressed in these cells.

The study shows that the Namalva cell line, which produces higher levels of interferon after induction with Sendai virus than Daudi cells, expresses the c-*myc* oncogene at a lower level than do the Daudi cells. However, Namalva cells express the c-Ha-*ras* genes at levels similar to those of Daudi cells. The translocation of the c-Ha-*ras* oncogene but not the c-*myc* oncogene might have occurred in Namalva cells. Another explanation for a reduced level of c-*myc* expression in Namalva cells is the unique feature of this cell line as an interferon producer, since it has been shown that interferon reduces oncogene expression (25, 30, 62, 93).

Two important areas of study in cancer research are tumorigenesis and treatment. Knowledge of interferon mRNA production and interferon protein formation *in vitro*

provides a basis for the further study of interferon therapy *in vivo*. In case of Namalva cells such information might lead to more successful applications in cancer therapy if one decides to use such interferon for treatment of patients with Burkitt's lymphoma. Thus, the work on oncogenesis helps to shed light on the understanding of tumorigenesis, at least in Burkitt's lymphoma derived cell lines.

The two parts of this study, i.e., interferon genesis and expression of oncogenes, are interrelated since interferon is used in cancer therapy. Furthermore, the work has provided additional information on the expression of oncogenes in human lymphoblastoid cell lines and on the expression of IFN-α mRNA in the same system.

APPENDIX

APPENDIX

1a. SSC (1X):

Sodium chloride	8.2 g
Sodium citrate	4.4 g
Water	to one liter

*All water used was double distilled deionized

1b. The same as 1a but it is 10 times less concentrated.

1c. The same as 1a but it is two times more concentrated.

1d. The same as 1a but it is six times more concentrated.

1e. The same as 1a but it is 10 times more concentrated.

1f. The same as 1a but it is 20 times more concentrated.

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2. Oligo(dt) binding buffer:

Sodium chloride	29.22 g
Tris (pH 7.5)	1.5 g
EDTA	0.37 g
SDS	5. g
Water	to one liter

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3. Oligo(dt) elution buffer:

Tris (pH 7.5)	1.5 g
EDTA	0.37 g
SDS	2. g
Water	to one liter

4a. Loading buffer:

SDS	0.5 g
Bromphenol blue	0.25 g
EDTA	0.93 g
Glycerol	25% (v/v)
Water	to 100 ml

4b. Loading buffer:

Ficoll	35. g
SDS	0.5 g
Bromphenol blue	0.1 g
Xylencyanol	0.1 g
Water	to 100 ml

5a. Peacock's buffer (1X):

Tris	10.8 g
Boric acid	5.5 g
EDTA (Na_2)	0.93 g
Water	to one liter

5b. The same as 5a but it is 10 times more concentrated.

---

6. Glyoxal treatment mixture:

Dimethyl sulfoxide	50% (v/v)
Glyoxal (stock of 40% w/v in water)	13% (v/v)
Sodium phosphate buffer (pH 6.5)	12% (v/v)
RNA or DNA at appropriate concentrations in water	25% (v/v)

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7. Methylene Blue (0.2% w/v) Gel Stain:

Methylene blue, 0.2 g, was added to 100 ml of 0.2 M sodium acetate-acetic acid buffer at pH 5.1

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8. Formaldehyde treatment mixture:

3-[N-Morpholino]propanesulfonic acid (MOPS) buffer:	10% (10X MPOS buffer is: MOPS (pH 7.0, 41.86 g, sodium acetate 6.8 g, EDTA 0.03 g, Water to one liter, Add 0.01% (v/v) of diethylpyrocarbonate to MOPS buffer at 37°C for 15 minutes and autoclave).
Formaldehyde (stock of 37%)	17.5% (v/v)
Formamide	50% (v/v)
RNA	22.5% (v/v)

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9. Preparation of composite gel:

Composite gels were prepared by mixing 100 ml of solution A (0.8 g agarose and 100 ml of water), 53 ml of solution B (16 ml of a 19 percent (v/v) acrylamide solution, 1 percent N,N'-methylene bis-acrylamide solution in water, 16 ml of Peacock's buffer (Appendix, #5a), 1 ml of 10 percent (v/v) N,N,N',N'-tetramethylethylenediamine and water to total volume of 53 ml, and 7 ml of solution C (1.6 percent (w/v) aqueous ammonium persulfate). These were mixed as follows: Solution A was boiled and cooled to 53° C and then mixed with solution B which was kept at 42° C. The mixture of solutions A and B was cooled down to 47°C and solution C then added.

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10. Reaction mixture used in end labeling (5'):

Polynucleotide kinase	2 to 3 units (0.5 µl)
Oligomer	5 to 500 ng (1 µl)
ATP[γ-^{32}P]	20 to 40 µCi (approximate specific activity of 5,000 to 7,000 Ci/mmol) (4 µl)
Universal buffer	9.5% (v/v) 1 µl (121.14 g Tris, 20.33 g magnesium chloride in one liter of water).
Water	to 10.5 µl

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11. Reaction mixture used in primer extension:

Template DNA (43)	2.4 µg
Primer (116)	5 ng
dCTP, dGTP, dTTP	2.4 µg each
DNA polymerase (Klenow fragment)	about 8 units
dATP[α-32P]	in sufficient quantity to give approximately 2×10^7 CPM (specific activity of 800 Ci/mmol)
Universal buffer	10% (v/v, appendix #10)
Water	to 50 µl

12a. Reaction mixture used in nick translations:

DNA	0.5 µg
Tris (pH 7.5)	188.25 µg
Bovine serum albumin	1. µg
Magnesium chloride	254. µg
dATP, dTTP, dGTP	150. ng each
Deoxyribonucleic acid Degrading enzyme (DNase)	0.1 ng
dCTP (α-^{32}P)	100 µCi (specific activity of 3,200 Ci/mmol)
DNA polymerase I	0.5 unit
Water	to 32 µl

12b.

DNA	0.5 µg
Tris (pH 7.5)	188.25 µg
Bovine serum albuin	1. µg
Magnesium chloride	25.4 µg
dTTP, dATP, dGTP	approximately 150 ng each
DNase	0.1 ng
dCTP(α-^{32}P)	80 to 100 µCi (specific activity of 2,800 Ci per mmol)
DNA polymerase I	0.5 unit
Total reaction mixture	25 µl

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13. Tris-EDTA (TE, pH 8.0):

Tris (pH 8.0)	1.41 g
EDTA (pH 8.0)	0.37 g
Water	to one liter

14a. <u>Hybridization buffer</u>:

NET	6X (10X NET is 8.76 g sodium chloride, 2.25 g Tris, Hcl pH 7.5, 0.37 g EDTA (NA_2) pH 7.5 in 100 ml of water)
Denhardt's buffer	5X (100X Denhardt's buffer is 2 g ficoll, 2 g PolyvinylPyrrolidone, and 2 g bovine serum albumin pentax fraction v in 100 ml of water)
Sodium pyrophosphate	0.44 g
SDS	0.5 g
Water	to 100 ml

14b. Denhardt's solution 5X

SSPE	5X (20x SSPE is 210g sodium chloride, 27.6 g sodium phosphate (monobasic), 7.5 g EDTA pH 7.4 in one liter of water)
SDS	0.1 g
Denatured salmon sperm DNA (4)	10 mg
Formamide	50% (v/v)
Water	to 100 ml

14c. Denhardt's solution 5X

SSPE	5X
SDS	0.1g
tRNA or denatured Salmon sperm DNA	10 mg
Formamide	50% (v/v)
Water	to 100 ml

14d. Denhardt's solution 1X

SSC	4X
SDS	0.1 g
Denatured salmon sperm DNA	10 mg
Sodium phosphate buffer (pH 7.0, stock of 1M)	5% (v/v)
Formamide	50% (v/v)
Water	to 100 ml

15. Luria broth:

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g
Water	to one liter

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16. Lysing buffer:

Sucrose	25. g
Tris (pH 8.0)	0.7 g
Water	to 100 ml

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17. Triton-X-100 mixture:

Tris (pH 8.0)	0.7 g
EDTA	2.23 g
Triton-X-100	2% (v/v)
Water	to 100 ml

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18. Quick-blot reaction mixture:

Reagent A:	Brij 35	1 µl
Reagent B:	Sodium deoxycholate	1 µl
Reagent C:	Super saturated NaI	20 µl
Reagent D:	NaI-"plus" solution	50 µl

Pure nitrocellulose paper from Schleicher and Schuell Company is designated mRNC nitrocellulose paper.

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19. Acetic anhydrid solution:

Acetic anhydride solution	0.24% (v/v)
Triethanolamine	to 100 ml (from the stock of 0.1 M)

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