INTERFERON GENE EXPRESSION IN VIRUS-INDUCED HUMAN B-LYMPHOBLASTOID CELLS

by

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CONTAINS

PULLOUTS

to Isabel, for her endless patience and support

SUMMARY

Virus infection of human cells induces the transient expression of interferon-α and interferon-β. This thesis presents the results of investigations into the expression of these interferons in the human B-lymphobastoid cell line, Namalwa, following induction by Sendai virus.

Quantitative and qualitative changes in the synthesis of these interferon mRNAs and proteins were investigated in order to determine how interferon gene expression is controlled. Interferon mRNA was assayed both by translation in Xenopus oocytes and by hybridization with cloned interferon cDNA. Interferon was measured both by bioassay and by immuno-radiometric assay using a monoclonal antibody to interferon-α. In addition the effects of various treatment which perturb the normal control of interferon production have been assessed.

Sendai virus infection of Namalwa cells resulted in the coordinate induction and regulation of both interferon-α and interferon-β synthesis. Although significant amounts of interferon-β were present, the cells did not produce any functional interferon-β protein. It was concluded that the interferon-β mRNA in these cells was inactive.

Production of interferon was shown to be increased by incubating cells at reduced temperatures following induction. It was concluded that the normal inactivation and degradation of interferon mRNA which occurs during the shut-off of interferon production was inhibited at the lower temperature. This resulted in increased interferon production over a prolonged period.

Treatment of cells with butyrate or 5'-bromodeoxyuridine before induction caused a dose-dependant increase in the rate of interferon mRNA and interferon synthesis. These treatments appear to coordinately affect the control of both interferon-α and interferon-β gene expression, since no differences could be detected in the characteristics of interferon or interferon mRNA produced by treated and untreated cells. The effect of these treatments was relatively specific, since polyacrylamide gel electrophoresis of proteins from butyrate- and 5'-bromodeoxyuridine-treated cells failed to detect any changes which were comprable to or could account for the effect on interferon synthesis.

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DECLARATION

The results presented in this thesis were obtained entirely by myself, with the following exceptions. The experiments described in section 3.1 were performed in collaboration with Dr.J.Morser who provided the data shown in Figs,6,7,8 and 9 and Table 5. The data in Table 12 were also provided by Dr.J.Morser.

The results in sections 3.1, 3.2 and 3.4 have now been published and copies of these papers are included at the end of this thesis.

ABBREVIATIONS.

A260/280	- absorbance at 260nm/280nm
BrdUrd	- 5'-bromodeoxyuridine
BSA	- bovine serum albumen
cDNA	- complementary DNA
Ci	- curie
cpm	- counts per minute
DNA	- deoxyribonucleic acid
DNase	- deoxyribonuclease
DRB	- 5,6-dichloro-1-β -D-ribofuranosylbenzimidazole
EBTr	- embryonic bovine tracheal cells
EBV	- Epstein-Barr virus
EDTA	- ethylenediamine tetraacetate
GMEM	- Glasgow modification Eagles medium
HFF	- human foreskin fibroblasts
hnRNA	- heterogeneous nuclear RNA
IEF	- isoelectric focussing
IFN	- interferon
Kb	- kilobase
MDBK	- Malin-Darby bovine kidney cells
Mr	- relative molecular weight
mRNA	- messenger RNA
NDV	- Newcastle disease virus
NEPHGE	- non-equilibrium pH gradient gel electrophoresis
NP40	- nonidet P 40
poly(A)	-polyadenylate
PBS	- phosphate buffered saline
poly(rI).poly(rC)	- polyriboinosinic acid.polyribocytidylic acid
PVP	- polyvinylpyrrolidone

RNA	- ribonucleic acid
RNase	- ribonuclease
RNP	- ribonucleoprotein
SDS	- sodium dodecyl sulphate
SFV	- Semliki Forest virus
SV 40	- Simian virus 40
TCA	- trichloroacetic acid
tris	- tris(hydroxymethyl)aminoethane

SECTION 1 INTRODUCTION

Section 1.1 Regulation of eukaryotic gene expression

Studies on the regulation of gene expression in eukaryotic cells have in recent years indicated that a complex array of regulatory mechanisms are involved in controlling the expression of genetic information and in determining the structural and functional phenotype of a cell. This complexity reflects the variety of situations in which different genes are required to be expressed, for example during differentiation and specialisation, in response to physiological stimuli (for example hormones) or during normal cell growth. The interferon (IFN) genes are a group of inducible structural (protein coding) genes which are expressed in cells exposed to a number of stimuli, such as virus infection. This thesis presents the results of studies on the expression of these genes in a cultured human cell line. Before describing the IFN genes and their expression it would be useful to briefly consider current ideas about the regulation of eukaryotic structural gene expression in order to provide a framework for later discussion.

Regulation of gene expression can broadly be considered to involve all these processes which quantitatively or qualitatively cause variation in gene activity and gene products. With few exceptions (see below) the molecular basis of the events which cause this variation is not known. However studies on the structure of individual viral and cellular genes and the transcription and processing of their mRNAs have indicated that in eukaryotes gene expression is regulated at a variety of levels (reviewed by Brown, 1981; Darnell, 1982), unlike prokaryotes where control occurs exclusively at the level of transcription initiation and termination

(Miller and Reznikoff, 1978). Fig. 1 shows a schematic repr esentation of the events leading from gene-mRNA-protein. The levels at which control can occur have been divided into four categories for the purpose of discussion, these are a) transcriptional, involving the interaction of RNA polymerase with DNA, b) post-transcriptional, including formation of mRNA from $_{hn}$RNA transcripts and its stability in the cytoplasm, c) translational, involving the utilisation of mRNA in the polyribosomes and d) post-translational in which the modification and turnover of protein are controlled. The processes occurring at each level need not themselves be regulatory since they are in most cases constitutive and therefore only mediate the variation in gene expression. This section is intended to describe these potential control points and to discuss information supporting their involvement in the regulation of gene expression.

a) <u>Transcriptional control</u>

Control at the transcriptional level involves those mechanisms which direct the transcription of RNA from the DNA template. Information about these mechanisms has been derived by three different approaches; i) studies on gene structure, ii) studies on the organisation of chromatin and iii) measurements of the transcriptional activity of genes. The important facts to emerge from these studies are summarised below.

i) Gene structure

The benefits to be gained from an understanding of the primary structure of individual genes are self evident. A sufficient number of structural genes have now been isolated and sequenced to arrive at what could be considered a consensus polymerase II transcription unit. Fig. 2 describes the essential features of such a unit encoding a single mRNA and protein.

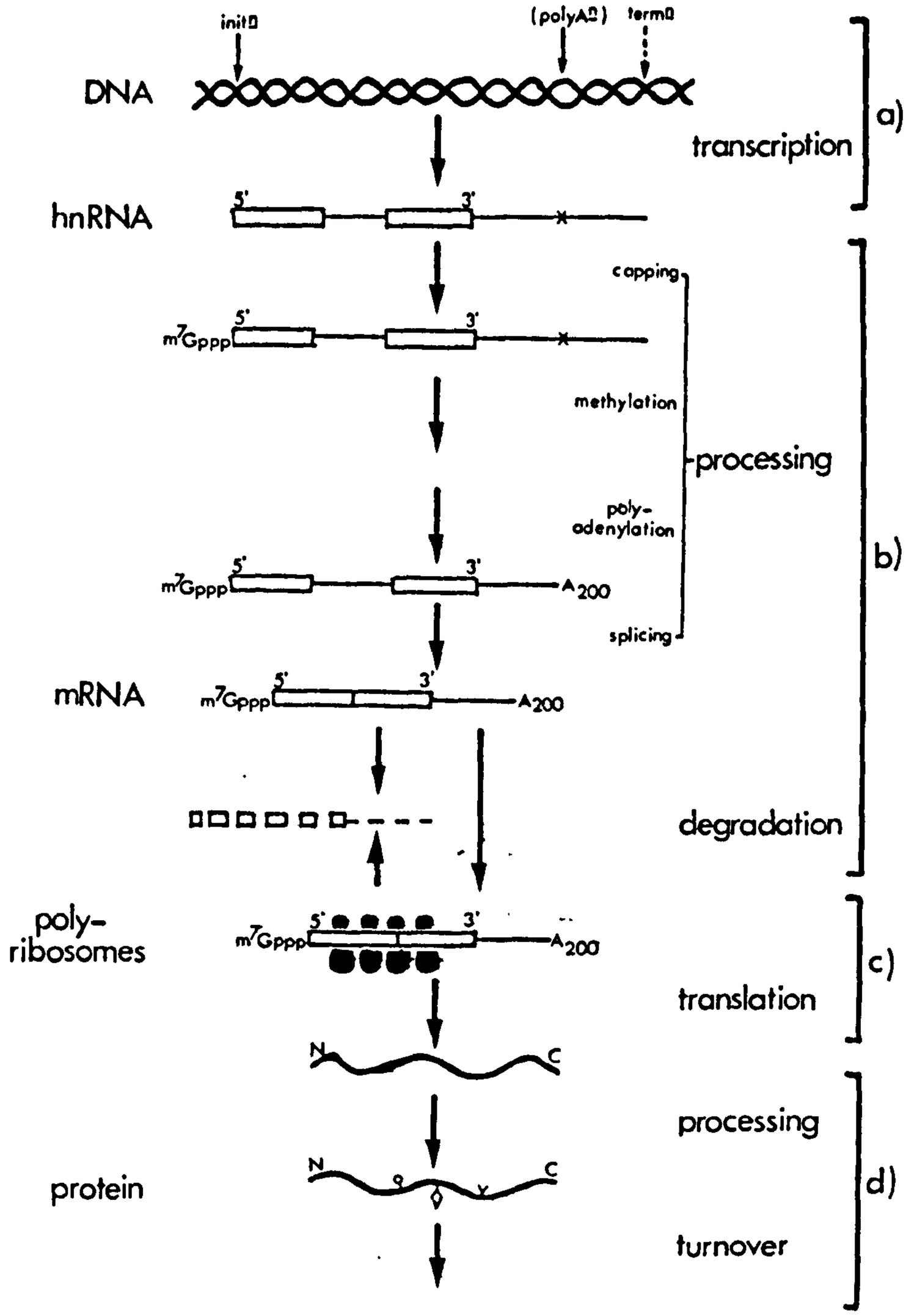


Fig. 1. The levels at which gene expression may be controlled.

a) transcription, b)post-transcription, c) translation,

d) post-translation.

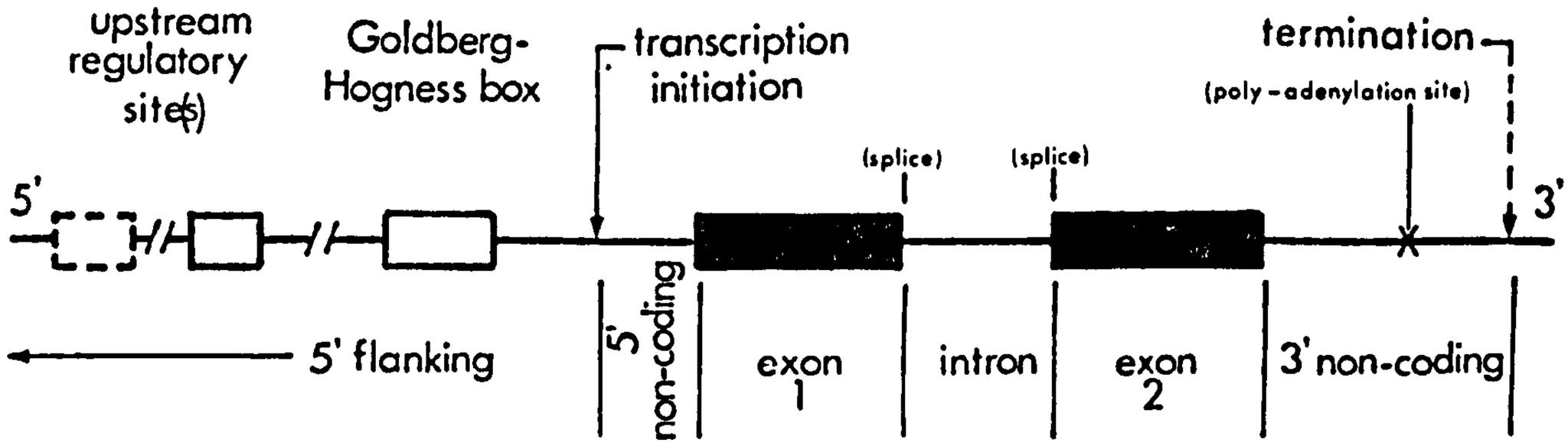


FIG.2. The structural features of an RNA polymerase II transcription unit.

The positional information which is required to select accurate transcription is provided in most genes by a conserved 8-10 nucleotide sequence known as the Goldberg-Hogness box (Goldberg, 1979), in which the sequence T-A-T-A usually occurs. Transcription is initiated at a site located 25-30 nucleotides downstream (in a 3' direction) from the Goldberg-Hogness box (Grosschedl and Birnstiel, 1980). Transcription then proceeds through protein coding and non-coding regions of the gene and terminates in some 3' region which has yet to be described. Increases and decreases in the rate of transcription of a gene are thought to be mediated by varying the efficiency with which RNA polymerase II interacts with the selector and initiator sequences described above. In vitro, site directed mutagenesis of cloned genomic DNA and its transcription in surrogate in vivo systems such as Xenopus oocyte nuclei have indicated sequences further upstream from the Goldberg-Hogness box which are important in modulating the rate of transcription in several genes, including the sea urchin H2A histone genes and the Herpes tK gene (Grosschedl and Birnstiel, 1980;

McKnight and Kingsbury, 1982). No clear indication of the role of such 5' sequences has yet been discovered, but it has been proposed that they may be involved in DNA-protein interactions which alter the efficiency of RNA polymerase II binding and initiation of transcription (McKnight and Kingsbury, 1982). The identity and functional role of these transcriptional signals are being investigated by the techniques of gene transfer. Cloned DNA containing protein coding genes and their flanking regions have been introduced into heterologous cells by calcium co-precipitation (Wigler et al., 1979) and shown to carry all the information required for regulated expression. For example, hormonal regulation of the rat $\alpha 2\mu$ globulin gene (Kurtz, 1981) and the human growth hormone gene (Robbins et al., 1982) has been demonstrated in transformed mouse cells. The first real indication that transcription may indeed be modulated by protein-DNA interactions has been provided by the glucocorticoid responsive murine mammary tumour virus genes. Activated glucocorticoid receptor protein binds selectively in vitro to a cloned fragment of murine mammary tumour virus DNA (Payvar et al., 1981; Ucker et al., 1982). However at present little is known about the transcriptional signals required for activation of genes during development and differentiation. Logically certain aspects of gene structure/organisation must be involved in the regulation and coordination of transcription of genes such as the highly reiterated histone genes (Hentschel and Birnstiel, 1981) and the globin gene clusters (Efstratiadis et al., 1980).

ii) The structure of active chromatin

There is now a considerable amount of evidence to suggest that the structure of chromatin from actively transcribed genes is different to that from non-transcribed regions of the genome.

All eukaryotic DNA exists in association with histones and other chromosomal proteins which serve not only to package the DNA and maintain higher orders of chromatin structure but also to regulate the accessibility of genes to transcriptional apparatus. The majority of the DNA in the nucleus is in the form of highly condensed chromatin and is transcriptionally inactive. However it has been found that active genes are preferentially sensitive to nuclease digestion, suggesting that the DNA is present in a more relaxed chromatin structure (Weintraub and Groudine, 1976). Following the original observation that the globin genes in erythrocyte nuclei are preferentially degraded by pancreatic DNase I , the observation has been extended to numerous other transcriptionally active genes (reviewed by Weisbrod 1982a). In addition limited DNase I digestion has identified hypersensitive regions located at or near the 5' end of active genes which possibly represent the regions involved in regulating and initiating transcription (reviewed by Elgin, 1981).

Several mechanisms appear to contribute to the changes in higher order chromatin structure which accompany gene activation. Modification of the DNA itself, in the form of methylation of cytosine residues, is thought to be associated with inactive/condensed chromatin, and indeed there is a degree of correlation between undermethylation, DNase I sensitivity and in vivo and in vitro transcription of some genes (Ehrlich and Wang, 1981; Weisbrod, 1982a). Modification of chromosomal proteins is also observed in transcriptionally active chromatin, for example acetylation of core histones, (histones H2A, H2B, H3 and H4 which are associated with nucleosomes) and phosphorylation of histone H1 accompany decondensation of chromatin (see below and Weisbrod, 1982a). In addition certain non-histone proteins such as HMG 14 and HMG 17 are preferentially associated with such domains and thought to be involved in events which facilitate transcription (Weisbrod, 1982b). However, DNase I sensitivity,

undermethylation and modification of chromosomal protein, although characteristic of active chromatin, only indicate the potential for transcription. The primary events which elicit these changes in chromatin structure and regulate its transcriptional capacity are not known.

iii) Measurements of transcriptional activity

Examples of apparent transcriptional control, resulting in increases or decreases in cytoplasmic mRNA, have been reported for numerous eukaryotic genes. However, steady-state levels of a mRNA can also be influenced by changes in its nuclear and cytoplasmic stability, therefore evidence of transcriptional control requires direct measurements of the rate of transcription. This can be estimated by measuring the incorporation of radioactive precursors into nascent nuclear RNA corresponding to a specific mRNA. Using such measurements, transcriptional control has been demonstrated for several genes. For example, stimulation of egg white protein production in the chicken oviduct by oestrogen (McKnight and Palmiter, 1978), glucocorticoid regulation of the mouse metallothionein gene (Hagar and Palmiter, 1981) and the switch from embryonic to adult globin gene expression in chick embryo erythoblasts (Weintraub et al., 1981). In addition the expression of several mouse liver specific mRNAs (ranging in abundance from 100- 10,000 copies/cell) appeared to be controlled at the transcriptional level (Dermann et al., 1981). Transcriptional control is therefore frequently used to regulate the expression of a wide range of genes.

b) <u>Post-transcriptional control</u>

i) RNA processing

The utilization of primary RNA transcripts (or hn RNA) is

dependant on successful processing to form mature mRNA and its export from the nucleus. The sequence of events which convert hnRNA into mRNA are summarised in Fig. 1. The first of these events is the addition of a "cap" or 7-methylguanosine (m^7G) residue to the 5' terminus via a series of intermediate reactions(Perry, 1981). Most, if not all RNA polymerase II transcripts are modified in this way. The post-transcriptional addition of a poly(A) segment to the 3' end is a more selective process, only 25% of capped molecules being polyadenylated. This involves endonucleolytic cleavage and addition of 150-200 adenylate residues at a site indicated (at least partly) by an A-A-U-A-A-A sequence located within the 3' untranslated region of the transcript (see Fig. 2). Histone mRNAs are the only mRNAs known not to undergo polyadenylation (Darnell, 1982).

The protein coding regions (exons) of most eukaryotic genes are interrupted by non-coding sequences or introns, as indicated in Fig. 2. To date, the only genes known to lack introns are histone genes (Hentschel and Birnstiel, 1981) and the human IFN-α and IFN-β genes (see below). The removal of these intervening sequences or splicing is required to form functional mRNA with a continuous coding sequence. Following this final processing step the capped, polyadenylated and spliced mRNA leaves the nucleus.

The "decision" to process or discard a specific transcript provides an obvious control point by regulating the cytoplasmic mRNA levels and hence the expression of a gene. Such control must occur since the sequence complexity of nuclear, hnRNA is 10-20 times greater than that of cytoplasmic mRNA (Darnell, 1982). This difference cannot be accounted for by intron loss during processing, therefore a large proportion of primary transcripts must be degraded. Similar conclusions were drawn by comparing the fate of capped hnRNA molecules in Chinese hamster ovary cells (Salditt-

Georgieff and Darnell, 1982). Only 25-50% of primary transcript sequences were found in stable polyadenylated mRNA in the cytoplasm.

One example where differential processing could be involved in regulating gene expression is the growth stimulation of dihydrofolate reductase levels in methotrexate-resistant mouse cells (Leys and Kellems, 1981). It was found that the appearance of mRNA in the cytoplasm was dependant not on increased transcription but increased stability of transcripts in the nucleus.

ii) Control of mRNA stability

Very little is known about the control of mRNA stability in the cytoplasm. The mechanisms and structural features which control and determine the half-life of a mRNA remain obscure, although it is possible that the cap structure and poly(A) tail could function to prevent (or delay) exonuclease degradation of the molecule. It is clear that different mRNAs can have widely different stabilities in the same cell, for example the mRNA population of HeLa cells can be divided into two components based on the kinetics of their decay (following ^{3}H-uridine labelling). Both components decay exponentially, one with a half-life of around 4h or less, the other with a half-life greater than 24h (Lewin, 1980). What distinguishes each stability class is not clear, but it would appear that lower abundance mRNAs are closely associated with the less stable population. One consequence of a rapid decay rate would be that the cytoplasmic levels of a mRNA could be rapidly adjusted once its synthesis had stopped and its presence was no longer required. However the physiological significance of this variety in mRNA stability will only be explained by comparing the half-lives of specific mRNAs for proteins of known function.

Changes in the stability of a specific mRNA provide an additional

means of regulating cytoplasmic mRNA levels to meet the requirements of a cell. Several examples of variations in the half-life of a mRNA have been reported, these usually accompanying changes in the rate of its transcription. The accumulation of casein mRNA following prolactin stimulation of breast tissue is caused by a combination of increased transcription and mRNA stability. (Guyette et al., 1979). In contrast, the suppression of ∝- fetoprotein production in newborn rats by glucocorticoids results from decreased transcription and mRNA stability (Fu Chui et al., 1981).

c) Translational control

It has been recognised for a long time that mRNA present in the cytoplasm need not necessarily be involved in translation, the most striking example being provided by amphibian oocytes. For instance, more than 90% of the mRNA pool of Xenopus oocytes is not present in polyribosomes (Rosbach and Ford, 1974). It would appear that a large proportion of this inactive mRNA is present in a "masked" untranslatable form in mRNP particles, and can be activated during maturation or after fertilisation. Similar gross changes in translation of mRNA populations occur during the heat shock response and after virus infection of cultured cells (Darnell, 1982) which result in a general reduction of total protein synthesis.

d) Post-translational control

The function or biological activity of a protein is often dependant on post-translational modification, such as glycosylation, phosphorylation, and proteolytic cleavage, each of which could provide

a control point influencing the expression of a functional gene product. Processing of the polyprotein precursor known as pro-opiomelanocortin in the brain and pituitary is one example of a post-translational event known to perform a regulatory function (Herbert and Uhler, 1982). This large precursor polypeptide is cleaved at different points to produce either adrenocorticotropin, melanocyte stimulating hormones and lipotropin in the anterior pituitary, or endorphins in the intermediate lobe. This differential processing therefore performs a regulatory function, qualitatively determining the expression of peptide hormones.

e) Modulation of gene expression by drugs and metabolic inhibiters

Numerous indirect approaches have been used to investigate the regulation of gene expression, some of which involve the addition of agents which perturb normal cellular processes. One strategy, which is of particular relevance to the study of IFN gene expression is the use of inhibitors to block RNA and protein synthesis. The information that has been obtained about IFN production using this approach is discussed further in section 1.2. An alternative strategy employs agents which appear to selectively modulate the expression of specific gene products. The short chain fatty acid butyrate and the halogenated pyrimidine 5'-bromodeoxyuridine (BrdUrd) have also been used to study IFN production (see section 1.3) therefore it would be useful to review the effects of these agents on gene expression in general.

i) The effects of butyrate on gene expression.

Low concentrations of butyrate have a varied and well documented effect on the expression of many differentiated and specialised cell proteins (reviewed by Prasad and Sinha, 1976). For example, butyrate

increases the synthesis of human chorionic gonadotrophin and follicle stimulating hormone by HeLa cells (Ghosh and Cox, 1976; 1977), prostaglandin synthase activity in mastocytoma cells (Koshihara et al., 1981) and induces differentiation and haemoglobin accumulation in murine erytholeukemic cells (Leder and Leder, 1975). In contrast, butyrate inhibits steroid induction of chicken egg white proteins (McKnight et al., 1980) and glucocorticoid-induced tyrosine amino transferase in hepatoma tissue cultures (Tichonicky et al., 1981). The biochemical effects of butyrate are often accompanied by alterations in cell morphology (Simmons et al., 1975) and butyrate-treatment has been shown to cause reversion of the transformed phenotype of murine sarcoma virus-infected cells (Via at el., 1980).

It has been assumed that the effects of butyrate on gene expression are a reflection of altered patterns of transcription although direct evidence has not always been obtained. The butyrate-induced biochemical and morphological alterations in HeLa cells are blocked by inhibitors of RNA and protein synthesis (Simmons et al., 1975; Fishman et al., 1975). Similarly butyrate-treated murine erytholeukemic cells and chick myoblasts accumulate new unique-sequence RNA transcripts and proteins (Reeves and Cserjesi, 1979; Fishman et al., 1980; Mintz et al., 1981). It would appear that butyrate acts by potentiating or initiating events which lead to these alterations in transcription and gene expression. This suggestion is based on studies of the effect of butyrate on chromatin structure and cell cycle progression. Butyrate effectively inhibits DNA synthesis and arrests cells in late G1 phase, close to the GI/S boundary (Hagopian et al., 1977; Fallon and Cox, 1979; D'Anna et al., 1980). Concomitantly butyrate also induces massive hyperacetylation of core histones, in particular H3 and H4, and also causes dephosphorylation of H1 and H2A (Vidali et al., 1978; Sealy and Chalkley, 1978; D'Anna et al., 1980). It is not clear

whether the effects of butyrate on cell cycle progression are a cause or a consequence of these histone modifications; H1 phosphorylation in particular is required for the G2 to M transition and also for entry into S phase. However it is known that butyrate inhibits the enzyme histone deacetylase in vitro (Vidali et al., 1978). By weakening electrostatic DNA-histone interactions, these changes in histone modification would be expected to favour chromatin dispersal and DNA accessibility, potentiating its template functions. In fact rapid, sequential acetylation/deacetylation in contiguous arrays of nucleosomes has been proposed to perform part of a surveillance mechanism during normal cell growth, allowing access for DNA repair and regulatory proteins (Perry and Chalky, 1982).

Direct evidence of changes in chromatin structure has been provided by comparing the DN ase I sensitivity of chromatin from butyrate-treated and untreated cells (Vidali et al., 1978). Chromatin from butyrate-treated cells was more sensitive to nuclease digestion (a property of transcriptionallly competent or active chromatin, see above), which is consistent with the idea that butyrate increases or potentiates transcription. Although there appears to be some correlation between histone hyperacetylation, increased DNase I sensitivity and increased RNA synthesis (Davie and Candido, 1980; Dobson and Ingram, 1980), histone modification per se is not sufficient to allow gene activation and transcription (Weisbrod, 1982a). In addition, whereas butyrate-induced histone acetylation and inhibition of DNA synthesis occur rapidly, and are maximal within 6-12h, changes in gene expression occur over a longer time scale of 24h or more. Therefore the effects of butyrate on gene expression must also involve other mechanisms which have yet to be identified.

ii) The effects of BrdUrd on gene expression

Incorporation of the thymidine analogue BrdUrd into cellular DNA

affects the expression of differentiated functions in a variety of cells. Treatment of cells with low concentrations of BrdUrd increases the synthesis of various enzymes and proteins such as glucose-6-phosphate dehydrogenase and prolactin in rat pituitary tumour cells (Bick and Soffer 1976; Biswas et al., 1977). BrdUrd and other halogenated pyrimidines also induce endogenous RNA and DNA tumour viruses (Besmer et al., 1975; Hampar et al., 1972). In constrast, BrdUrd inhibits differentiation in rat exocrine pancreas and DMSO induced murine erytholeukemic cells (Walther et al., 1974; Ashman and Davidson, 1980). All of these effects are reversed or prevented by the addition of thymidine which blocks incorporation of the analogue into DNA. However some of the effects of BrdUrd do not appear to depend upon its incorporation into DNA, for example the suppression of pigmentation in hamster melanoma cells (Davidson and Kaufman, 1977). This suppression can be reversed by the addition of deoxycytidine without reducing the amount of BrdUrd substituted into DNA. High concentrations of BrdUrd are known to inhibit the enzyme ribonucleotide reductase, therefore it could be that this effect of BrdUrd is due to its cy toxicity. However suppression of melanin synthesis in BrdUrd-treated murine melanoma cells has been shown to be accompanied by new mRNA and protein synthesis and is therefore not purely an-inhibitory effect (Price, 1976).

The mode of action of BrdUrd is not understood, although its substitution into DNA is known to increase the thermostability of chromatin and the binding affinities of chromosomal proteins (Lapeyre and Bekhor, 1974; Gordon et al., 1976; Schwartz, 1977). It has therefore been suggested that BrdUrd affects transcription by altering chromatin structure and perturbing the regulatory functions of chromosomal proteins. BrdUrd incorporation would be expected to promote chromatin condensation and antagonise the effects of agents

such as butyrate and DMSO which relax chromatin structure and are potent inducers of differentiation. This could account for some of the inhibitory effects of BrdUrd. However, as with butyrate, gross perturbation of chromatin structure is an unlikely explanation for the selective effects which these agents have on gene expression. Both agents undoubtedly affect the other cellular functions which could equally be involved in mediating their effects; firstly the butyrate-induced alterations in chromatin structure are not the direct cause of changes in gene expression and secondly, BrdUrd can affect transcription without being incorporated into DNA. Nonetheless butyrate and BrdUrd are useful tools for modulating gene expression and their use in studying the regulation of IFN synthesis is discussed below.

f) Summary

It would appear that control of transcription provides the most frequent (and fundamental) means of regulating eukaryotic gene expression. At present however, information is limited to a small fraction of the genes which are being expressed by a cell. A typical animal cell contains greater than 10^5 mRNA molecules (Davidson and Britten, 1979). Genes coding for abundant mRNAs such as globin and ovalbumin, which are present at more than 10^4 mRNA molecules/cell, have until recently been the major source of information about control of gene expression. Although genes coding for moderately abundant mRNAs (represented by a few hundred mRNA molecules/cell) have now been shown to be transcriptionally controlled (Dermann et al., 1981), the expression of genes represented by low abundance mRNAs (only 1 to several copies/cell) has not been investigated. One possible exception to this is the dihydrofolate reductase gene whose expression became accessible to study only after a 500-fold

amplification of gene copy number by methotrexate (Leys and Kellens, 1981). The finding that the dihydrofolate reductase gene was controlled post-transcriptionally leads to the speculation that other genes are controlled in this way. Indeed the significant difference between mRNA and hnRNA sequence complexity would suggest that a large number of genes are transcribed but not expressed. Therefore regulation of gene expression at levels other than transcription may play a more important role than appears at present.

To conclude, there is considerable scope for study of eukaryotic gene expression. Only by comparing the control of expression of a variety of genes, which are expressed in different cell-types and different circumstances, can a representative view of eukaryotic regulation be obtained.

Section 1.2 IFN gene expression.

a) Preamble

Interferon (IFN) is the collective name given to a group of functionally related proteins which are produced by cells in response to a variety of (seemingly unrelated) stimuli. These stimuli include viruses, natural and synthetic polynucleotides and, in the case of cells from the immune system, mitogens and specific antigens. Binding of IFN to responsive cells induces resistance to virus infection (the antiviral state) and also initiates a number of alterations in cell structure and function.

The IFNs produced by different combinations of inducer and cell-type are antigenically distinct. At present human and mouse IFNs are classified on the basis of their antigenic specificities (Committee on interferon nomenclature, 1980) into three main types, these being i) IFN-α (formerly called type I or leukocyte IFN) which is the major product induced in leukocytes by virus infection, 2) IFN-β (formerly called type I or fibroblast IFN) which is produced predominantly by virus infected fibroblasts, and 3) IFN-γ (originally type II or immune IFN) which is induced in lymphocytes by mitogen or antigen stimulation (reviewed by Stewart, 1979; Epstein, 1981).

Although cells may produce predominantly one type of IFN, often small quantities of another IFN type are also induced. Thus IFN induced in fibroblasts by NDV infection contains not only IFN-β but also up to 20% IFN-α (Hayes et al., 1979). Similarly, virus induced human B-lymphoblastoid cells, the subject of this thesis, are reported to produce a mixture of both IFN-α and IFN-β (Havell et al., 1977). It is unlikely (but as yet not proven) that IFN-γ is present in

virus induced IFN mixtures since the inducers and kinetics of induction of IFN-γ are very different to those of IFN-α and IFN-β (reviewed by Epstein, 1981). In addition, the gene coding for human IFN-γ is structurally unrelated to the human IFN-α and IFN-β genes which themselves show significant homology in both coding and flanking regions (Gray and Goeddell, 1982). It would appear that despite showing similar biological properties, the control of induction and expression of IFN-γ and the IFN-α/IFN-β family are completely different. For these reasons this introduction will be restricted to the human IFN-α and IFN-β gene family. Before discussing these genes and their expression, it would be useful to briefly consider some of the biological and physicochemical properties of human IFN-α and IFN-β to put into context the information in the remainder of this section.

The purification and characterisation of human IFNs from virus induced cells has clearly indicated that cells produce extremely small quantities of IFN. For example, only 1 μg of IFN-α was obtained by induction of leukocytes from 2 litres of human blood. (Rubenstein et al., 1979). Such purified preparations of IFN-α and IFN-β have been found to have very high specific activities, in the order of 2×10^8 units/mg of protein (Rubenstein et al., 1979; Zoom et al., 1979; Knight, 1976), where 1 unit is defined as the quantity of IFN which reduces virus replication by 50% in a specified assay. IFN-α and IFN-β activities have been found to be resistant to low pH conditions and (to varying extent) SDS and reducing agents such as β- mercaptoethanol. This has allowed their characterisation by polyacrylamide gel electrophoresis. IFN-α preparations show considerable size heterogeneity composed of molecules with apparent molecular weights ranging from 15,000-22,000 (Reviewed by Stewart, 1979). This heterogeneity was originally attributed to varying degrees

of glycosylation or post-translational modification. However, it is now clear, from gene cloning and amino acid sequencing (see below), that human IFN-α is composed of a family of structurally related proteins, all of which are equivalent in size and apparently non-glycosylated. The heterogeneity seen on polyacrylamide gels must presumably be artifactual. Surprisingly though, electrophoresis indicated differences in the composition of IFN-α preparations from human leukocytes and lymphoblastoid cells (Pickering et al., 1980). To accommodate these (as yet undefined) differences, IFN-α mixtures are also described by their cell-type of origin (lymphoblastoid, Ly; leukocyte, Le. Interferon committee on nomenclature, 1980). IFN-β is more homogeneous in size than IFN-α, with an apparent molecular weight of 20,000 on polyacrylamide gels (Knight et al., 1980). This is consistent with the fact that only a single human IFN-β gene and protein have been found (see below).

Human IFN-α and IFN-β can be distinguished not only by their antigenic specificity but also by their biological activity in cells from another species. IFNs in general are species-specific in their action, and indeed human IFN-β has virtually undetectable cross-species activity. IFN-α however, shows significant antiviral and growth inhibitory activity in bovine, murine, and feline cell lines (reviewed by Stewart, 1979).

The diversity of biological effects which have been attributed to purified IFN, <u>in vivo</u> and <u>in vitro</u>, suggest that it may play an important regulatory role. A detailed account of the range of effects and mechanism of action of IFN would be inappropriate here. However, from the point of view of defining the context in which IFN is normally expressed, the proposed regulatory functions of IFN can be summarised as follows. i) IFN has been shown to inhibit virus replication, inhibit normal and transformed cell growth,

cause changes in cell surface properties and cellular morphology, inhibit antibody production, and enhance surface antigen expression and natural killer cell recruitment (reviewed by Taylor-Papadimitriou, 1980). In an attempt to rationalise these varied effects it has been proposed that IFN acts as an effector molecule which is involved in host-defence mechanisms and immunologic surveillance. Thus IFN would be produced in response to a number of antigenic stimuli (for example viruses) and el icits a number of responses which limit or remove the foreign antigen.

ii) It has also been suggested that the growth inhibitory effect of IFN might be involved in the regulation of cell growth and differentiation. This is based largely on indirect evidence indicating that mechanisms involved in mediating the biological effects of IFN (reviewed by Baglioni, 1979; Lengyel, 1981; 1982) may also be involved in controlling cell growth during normal proliferation or differentiation (Stark et al., 1979; Kimchi et al., 1981). It is proposed that IFN is released by cells upon reaching confluence or at the onset of differentiation, preventing further cell divisions.

Consequently, at present no clear definition of the role of IFN can be made. Consideration of the control of IFN gene expression is therefore restricted largely to a description of model <u>in vitro</u> systems. Recently our knowledge of the IFNs has been considerably advanced by the cloning of the human IFN genes. The remainder of this section reviews our current understanding of the human IFN-α and IFN-β genes and the regulation of their expression <u>in vitro</u>.

b) <u>The human IFN-α and IFN-β genes and their expression</u>

The cloning of multiple IFN genes from human cDNA and genomic DNA, using recombinant DNA techniques, has indicated that IFN-α and IFN-β belong to a family of proteins whose genes are structurally

related. IFN-α consists of a family of 15 or more genes, a large number of which are known to be expressed. Only a single more distantly related IFN-β gene has been positively identified so far.

The human IFN-α family

Cloned cDNA sequences complementary to human IFN-α mRNA were first isolated by two independant groups from cDNA libraries prepared from leukocytes and a myeloblastoid cell line, both of which are predominantly IFN-α producers (Nagata et al., 1980a; Goeddell et al., 1980a). It was not long before it became apparent that these cDNA libraries contained several IFN-α related sequences. Restriction enzyme analysis and nucleotide sequencing indicated that at least 8 distinct IFNαcDNAs were present in the library from the myeloblastoid cell line, these were classified as Le IFN-A to Le IFN-H by the group concerned (Goeddell et al., 1981). The nucleotide and predicted amino acid sequences indicated that these IFNs were closely related. Homology at the nucleotide level varied from 84-94% in the coding regions of the genes. This complexity of the IFN-α family was confirmed when no less than 8 IFN-αrelated genes, classified as IFN-α1 to IFN-α8, were detected in a gene bank constructed from human chromosomal DNA (Nagata et al., 1980b). Most of these genomic sequences have been correlated with the cDNA sequences (Goeddell et al., 1981), although small differences in sequence suggest that many may be in fact allelic varients. This means that the different nomenclature of the two groups can be partly reconciled (see Table 1). To date 13 non-allelic human IFN-α genes have been positively identified, together with 9 allelic varients and 6 pseudogenes which show varying degrees of homology to the potentially functional IFN-α genes sequence (Weissmann et al., 1982; Brack et al., 1982, Lawn et al., 1981b). In addition further weakly hybridising but as

Table 1. The nomenclature used to describe the closely related IFN-∝ genes, correlated on the basis of nucleotide sequence data (reproduced from Weißsmann et al.,1982).

	IFN-∝1	≃	IFN-D
	IFN-∝2	≃	IFN-A
	IFN-∝3[a)]	≃	IFN-F
	IFN-∝5	=	IFN-G
	IFN-∝6	≃	IFN-K
	IFN-∝7	≃	IFN-J
	IFN-∝8	≃	IFN-B
not correlated	IFN-∝4		IFN-C
			IFN-I

= means identical ≃ means allelic or very similar

[a)] this designation was assigned to the protein sequence of Zoon et al.,1980, only IFN-F has been sequenced.

yet uncharacterised sequences have been reported. Several of the DNA segments carrying IFN-∝ related sequences overlap, indicating that at least 3 strongly hybridizing and 3 weakly hybridizing genes are linked. This suggests that the IFN-∝ genes may be clustered (Brack et al., 1982), and since all of the IFN-∝ genes have been located on chromosome 9 using hybridization of specific probes with genomic DNA from mouse-human cell hybrids carrying human chromosome 9 this may be the case (Owerbach et al., 1981).

All of the IFN-∝ gene sequences, with the exception of IFN-∝2 (LeIFN-A) contain a 567 nucleotide coding sequence. By aligning the predicted amino acid sequences with NH_2-terminal sequences from mature IFN-∝ (Le) and (Ly) (Zoon et al., 1980; Allen and Fantes, 1980; Levy et al., 1980) it was shown that this could code for a

putative preIFN consisting of 166 amino acids with a 23 amino acid signal sequence characteristic of most secretory proteins (Mantei et al., 1980; Goeddell et al., 1980a). Cleavage of the signal peptide would give rise to a mature IFN with a calculated molecular weight of 19,390 which agrees closely with the size of purified IFN-α(Ly) estimated from polyacrylamide gel analysis (Allen and Fantes, 1980).

One surprising feature of the IFN-α genes is that they contain no introns. Comparison of cDNA and genomic sequences and R-loop mapping showed that neither the coding or flanking regions possess intervening sequences (Nagata et al., 1980b; Lawn et al., 1981b; Brack et al., 1982). Other features of the IFN genes resemble those described for other structural genes. Sequence motifs resembling the consensus Goldberg-Hogness transcription signal (Goldberg, 1979) are found approximately 30 nucleotides upstream from the mRNA cap site. The protein coding sequence is preceded by a 70 nucleotide 5' non-coding region (Nagata et al., 1980b) and followed by 242-442 nucleotides of 3'untranslated sequence (Goeddell et al., 1981).

At least 10 of the 13 authentic, non-allelic IFN-α genes are potentially functional and probably expressed in cells since they produce active IFN when translated in E. coli (Nagata et al., 1980b), or are represented in cDNA libraries (Goedell., et al., 1981). Several investigators have reported heterogeneity of IFNmRNA in cellular poly(A)$^+$RNA from induced Namalwa (lymphoblastoid) cells which could partly be accounted for by the multiplicity of IFN genes (Berger et al., 1980; Sagar et al., 1981; Sehgal et al., 1981a, 1981b). Most of the IFN-α mRNA was around 1.3Kb in size, the predicted size for a mature poly (A) tailed mRNA transcribed from the genes described above. However larger transcripts from

1.8-3.5 Kb in size were detected in both lymphoblastoid cells and leukocytes which did not hybridize with any of the IFN-∝ genes already cloned, even though they were translated into proteins characterised as IFN-∝ by biological activity and neutralisation by anti-IFN-∝ sera. The significance of these transcripts will not be established until they have been fully characterised.

There is now a large amount of evidence indicating that cells do produce a mixture of IFN-∝ proteins, apart from the potentially artifactual data obtained from polyacrylamide gel electrophoresis. The NH_2-terminal amino acid sequence of one of several leukocyte IFN-∝ components resolved by reverse flow high performance liquid chromatography (Rubenstein et al., 1979; Levy et al., 1980) has close homology with the predicted NH_2-terminal sequence of cloned IFN-∝2. Similar data (Zoon et al., 1980) shows homology between the major component of Namalwa (lymphoblastoid) IFN-∝ and cloned IFN-∝3. At least 5 distinct, related polypeptides have been distinguished in Namalwa IFN-∝ by sequencing of tryptic and chymotryptic peptides (Allen and Fantes, 1980). Two of these show homology to, and have the same amino acid deletion at positions 44, as IFN-∝2. There are also similarities between the remaining 3 components and IFN-∝1, although additional species may be involved. This clearly demonstrates expression of multiple IFN-∝ species, but also suggests that different cell-types (and inducers) may preferentially express different mixtures of IFN-∝ proteins, an idea supported by the frequency of IFNcDNA species found in libraries from different cell types (Nagata et al., 1980b; Goeddell et al., 1981). It seems unlikely that any of these IFN-∝ proteins are glycosylated since no sequence of the type Asn-X-(Ser or Thr), which is required for N-glycosidic linkage, occurred in any of the tryptic or chymotryptic peptides prepared from Namalwa IFN-∝ (Allen and Fantes,

1980). Furthermore this IFN had a very low amino sugar content, although neither observation eliminates the possibility of O-linked glycosylation or glycosylation of minor components.

The biological and physicochemical properties of several of the cloned IFN-α gene products expressed in E coli have been compared with natural HuIFN-α(Le) and have been shown to share all of the characteristics attributed to IFN-α. Cloned IFN-α induced virus resistance in vivo and in vitro, inhibited cell proliferation, modulated various immune responses, was neutralised by anti-IFN-α and was stable at pH2 (Goeddell et al., 1980a; Massuci et al., 1980). Another significant finding was that the individual cloned IFN-α species differed in their anti-viral activity in various heterologous cells (Streuli et al., 1981; Weck et al., 1981).

The high heterospecific activity of IFN-α species, particularly in bovine cells varied over an order of magnitude. This behaviour was predicted by the characterisation of polyacrylamide gel fractions of natural IFN-α mixtures when it was found that the fastest migrating components of IFN-α(Le) were significantly more active in bovine cells than in human cells (Stewart II et al., 1975). In fact cloned IFN-α1, the species with the highest ratio of activity in bovine compared to human cells, comigrated with the leading edge of IFN-α (Le) in polyacrylamide gel and had a similar molecular weight (18-19,000) and chemical properties (Stewart II., 1980). In conclusion it can be said that the multiplicity of human IFN-α genes is reflected in, and confirmed by, the expression of a family of IFN-α proteins. The E.coli synthesised cloned proteins have all of the structural, physiochemical and biological properties of natural IFN-α.

The human IFN-β gene

The cloning of IFN-β cDNA from cDNA libraries prepared from poly(rI).poly(rC)-induced fibroblasts was reported by several independent groups shortly after the first isolation of IFN-α cDNA clones (Taniguchi et al., 1980a; Goeddell et al., 1980b; Derynck et al., 1980; Houghton et al., 1980). Comparison of its coding sequence with that of IFN-α1 showed that in fact IFN-α and IFN-β were structurally related (Taniguchi et al., 1980b), with approximately 45% homology at the nucleotide level and 29% at the amino acid level. This indicated that IFN genes were most probably derived from a common ancestor.

Direct restriction analysis of the human genome using this IFN-β cDNA revealed that only a single IFN-β gene was present (Tavernier et al., 1981). The cloned IFN-β gene resembled IFN-α genes in several of its structural features. Firstly, neither the coding or untranslated sequences contained introns (Degrave et al., 1981). Secondly, the coding region gave a predicted pre-IFN-β protein 187 amino acids in length which when aligned with NH_2-terminal sequence data for mature IFN-β, indicated that the IFN-β protein was 166 amino acids in length, the remaining 21 amino acids forming the signal sequence (Knight et al., 1980; Derynck et al., 1980). Therefore the mature IFN-β polypeptide is of the same size (approximately 19,000) as IFN-α. However unlike IFN-α, IFN-β is most probably a glycoprotein, as was previously suggested (Tan et al., 1979), since its predicted amino acid sequence includes at least one Asp-X - (Ser or Thr) N-glycosidic linkage site. The predicted amino acid sequence from all of the IFN-β cDNA and genomic clones and the published NH_2-terminal sequence of fibroblast produced IFN-β were all in close agreement, which strongly suggested that only a single IFN-β was present in the human genome. A second IFN-β cDNA was thought to have been isolated from

a mRNA which apparently showed IFN-β mRNA activity (Weissenbach et al., 1980). This cDNA did not hybridize with the existing IFN-β cDNA, and no further characterisation has been reported. Other groups have failed to detect the mRNA used to construct this cDNA (Content et al., 1982).

Further similarities between IFN-β and IFN-α1 genes occur in their 5' flanking regions. In addition to the Goldberg-Hogness box at position -31 other consensus sequences appear further upstream from the cap-site (Degrave et al., 1981). Two of these motifs are similar to those found in other genes and one in particular occurs only in inducible genes (Benoist et al., 1980). The significance of these sequences for expression of IFN genes will obviously require further investigation. However, if such 5' flanking sequences are involved in regulating transcription then IFN-α and IFN-β genes would conceivably show homologies in those regions since they respond to the same inducer (see part C below). The IFN-β gene as with the IFN-α gene family, is located on chromosome 9 (Meager et al., 1979a; Owerbach et al., 1981), although it is not known how closely the genes are organised.

Further evidence that only a single IFN-β gene is present and expressed in human cells is provided by reports that IFN-β mRNA consists of a discrete population of molecules around 1.1 Kb in size (Raj and Pitha; Gross et al., 1982). However it has been suggested that no less than 5 IFN-β mRNAs can be detected under certain circumstances (Sehgal et al., 1981a). Two size classes of IFN-β mRNA were found in poly(rI).poly (rC) induced fibroblasts (Sehgal and Sagar, 1980). Although the IFN activity translated from both classes of mRNA was neutralised by anti-IFN-β serum, the larger transcripts did not cross-hybridize with the established IFN-β sequence. Further investigation using somatic-cell hybrid analysis located 4

of the 5 putative IFN-β genes to human chromosomes 2,5 and 9 (Sehgal et al., 1981a). These assignments are interesting since originally chromosomes 2 and 5 were implicated as being required for human IFN production (Tan et al., 1974). These results are perplexing since as with the large IFN-α transcripts (Sehgal et al., 1981b) it is difficult to reconcile the fact that these additional genes appear to show little sequence homology, while their proteins share antigenic characteristics with authentic IFN-β. This could only be accounted for by the presence of structurally unrelated genes (or perhaps related genes with introns) which code for proteins with structural features similar to IFN-α and IFN-β. If this is the case then their classification as IFN-α and IFN-β is debatable.

To conclude, IFN-α and IFN-β form a structurally related multigene family which codes for a group of proteins which share many biological and physicochemical properties.

c) <u>The regulation of human IFN-α and IFN-β gene expression</u>

The study of IFN-α and IFN-β gene expression in cultured human cells has shown that IFN synthesis is tightly regulated. Virus induction of cells results in a brief period of <u>de novo</u> IFN synthesis usually lasting only a few hours. This is followed by the rapid decline and shut-off of IFN synthesis. It would appear that the IFN genes are available for transcription (in the presence of inducer) in most cell-lines, although the IFN-α and IFN-β genes are differentially expressed in different cell-lines and with different inducers. There is some evidence to suggest that the mouse IFN genes are in a non-inducible or non-transcribable state in mouse teratocarcinoma cells and early mouse embryo tissues (Burke et al., 1978; Barlow, 1981). Virus infection failed to induce IFN synthesis in these cells. However,

similar information is not available for human cells.

Constitutive or "spontaneous" IFN production has been described in several lymphoblastoid cell-lines (Adams et al., 1975; Pickering et al., 1980). Although a number of these lines carry Epstein-Barr virus (EBV), no correlation was found between expression of EBV antigens and constitutive IFN production (Tovey et al., 1977; see also section 1.3). The IFN was neutralised by anti-IFN-α but did not show the characteristic size heterogeneity of induced IFN-α mixtures (Pickering et al., 1980). These cell lines respond normally to induction by producing increased levels of IFN, therefore the normal induction-regulation mechanisms are functional. Constitutive IFN synthesis could be an indication of "leaky" regulatory mutants or the presence of unidentified inducing stimuli. Alternatively, perhaps some or all cells constitutively express low levels of IFN as suggested by the "Basal-level IFN hypothesis" (see below).

Induction of IFN synthesis

Information about the inducer and mechanism of induction of IFN-α and IFN-β synthesis remains almost anecdotal, belying the fact that over 20 years of intensive research has been directed towards describing these aspects of the IFN system. One of the problems has been the diversity of stimuli which initiate IFN synthesis, no common inducer or sequence of events can be identified.

Representatives of every major virus family can induce IFN in human cells (Stewart II, 1979). These include single-stranded RNA, double-stranded RNA and DNA viruses. The effectiveness of individual viruses can vary depending on the strain of virus and the cell-type, however several viruses have emerged as being particularly good inducers of IFN synthesis in a variety of cell-types, notably paramyxoviruses such as Newcastle disease virus (NDV) and Sendai virus (Ho et al., 1970;

Gresser, 1961). In describing a virus as an inducer of IFN synthesis it should be remembered that any one of the events involved in its replication is potentially capable of providing the stimulus although adsorption, penetration and uncoating can be eliminated since infectious viral RNA alone is sufficient to induce IFN. The intrinsic ability of any of the remaining events to initiate IFN synthesis can be masked by the effects of virus replication on cellular macromolecular synthesis. Consideration such as these have made it difficult to define the nature of the proximate inducer of IFN synthesis.

Several synthetic inducers have been used to study the induction of IFN synthesis. By far the best (and most extensively studied) is the double-stranded polynucleotide, polyriboinosinic acid, polyribocytodylic acid or poly(rI).poly(rC). (Field et al., 1968). The discovery that it induces IFN synthesis prompted suggestions that double-stranded RNA was in fact the proximate inducer, since many viruses form double-stranded RNA during replication. Unfortunately research into the structural requirements of such a molecule for IFN induction failed to confirm a unique role for double-stranded RNA. For example, certain double-stranded polynucleotides were inactive, while single-stranded non-replicating viruses and poly(rI) could induce IFN (Torrence et al., 1975; Dianzani et al., 1974; Thang et al., 1977). Consequently no universal model can be proposed for the structure of the molecule which "triggers" IFN synthesis.

The induction mechanism

The initial interaction of the inducer with cells is followed by a lag period before the appearance of IFNmRNA or IFN. This lag or induction phase can vary depending on the combination of cell-type and inducer. For example human diploid fibroblasts induced by poly(rI).poly(rC) show a short 1-2h lag but NDV induction results in an 8h lag before the IFN production phase (Mozes and Vilcek, 1975).

The induction mechanism can therefore be considered to include all of the events which occur between the primary inducer-cell interaction and the eventual transcription and synthesis of IFNmRNA, and most likely consists of a sequential multi-step process.

Numerous mechanisms have been proposed to explain the events which give rise to IFN synthesis, but basically only three hypotheses can be considered to retain some credibility:

i) "The double-stranded RNA hypothesis" is an attempt to describe a unifying mechanism whereby double-stranded RNA, in the form of virus replicative intermediate or synthetic polynucleotides, triggers a sequence of events which initiate IFN synthesis. Indeed several lines of evidence indicate that early virus functions are required. For example UV-inactivation of NDV first causes loss of infectivity, then at higher doses, loss of polymerase activity. The capacity to induce IFN was destroyed in parallel with the loss of polymerase activity, suggesting that viral RNA synthesis and the formation of double-stranded replicative intermediates was required (Meager et al., 1972). Unfortunately, there is an equal amount of evidence arguing against the involvement of double-stranded RNA, for example the induction of IFN synthesis by single-stranded viral and synthetic RNA (see above). The wealth of evidence for and against this hypothesis is reviewed extensively by Stewart,(1979).

ii) "The repressor-depletion hypothesis" was formulated to explain the observation that cycloheximide and other reversible inhibitors of macromolecular synthesis could induce IFN synthesis (Tan and Berthold, 1977). The ability of the inducer to initiate IFN synthesis was suggested to be due to inhibition of synthesis of a short-half life repressor molecule which normally repressed IFN gene activity. Indeed,

many viruses and double-stranded RNA do depress cellular macro molecular synthesis but conversely several effective inducers do not.

iii) "The basal-level IFN hypothesis" is related to the repressor depletion hypothesis and suggests that the repressor is in fact IFN which is synthesised constitutively at a low level. Evidence in support of this idea comes from the surprising observation that IFN is bound selectively to both poly(rI) and poly(rU) immobilized on columns (De Maeyer-Guignard et al., 1977). The selective binding of basal-levels of IFN by virus input RNA replicative intermediates or synthetic polynucleotides would then remove the repression from the IFN genes. Further support for this hypothesis has not been put forward.

In conclusion there is no all inclusive explanation to account for the variety of information on IFN induction. Whatever the mechanism, induction causes the increased synthesis of both IFNmRNA and IFN, since various studies have shown that synthesis of IFN can be prevented by addition of actinomycin D (an irreversible inhibitor of DNA dependant RNA polymerases) during the induction phase or by addition of cycloheximide (which inhibits protein synthesis) during the production phase. The requirement for RNA synthesis was taken as an indication that induction results in the derepression of the IFN genes. Although this assumption is probably correct, to date there is no direct evidence that induction causes an increase in transcription as opposed to an increase in IFNmRNA processing.

The control of IFN production

In most cells the induction phase is followed by the appearance and rapid accumulation of IFN. After a relatively short period the

rate of IFN synthesis reaches a maximum then rapidly declines to undetectable levels. This shut-off of IFN synthesis occurs even in the continued presence of inducer. By far the most intensively studied model is the production of IFN-β by poly(rI).poly(rC) induced fibroblasts, in which IFN production is first detected (in the medium) within 1h of induction, peaks at 2.5-3h and is shut-off by 6-8h (Sehgal et al., 1977).

Investigations into the regulation of IFN production have made use of the effects of inhibitors of RNA and protein synthesis on the normal production of IFNmRNA and IFN in poly(rI).poly(rC) induced fibroblasts. Inhibition of RNA synthesis by actinomycin D indicated that IFNmRNA synthesis commenced shortly before the appearance of IFN and was complete by around 3h after induction, judged by the effect of the inhibitor on amounts of IFN produced (Sehgal et al., 1976; 1977). This suggested that IFN production was controlled at the transcriptional (or mRNA processing) level since IFNmRNA synthesis after 3h either did not occur or was not required. However, various combinations of reversible inhibitors of macromolecular synthesis, when added before the shut-off, prolonged IFN synthesis and resulted in a paradoxical increase or "superinduction" of IFN synthesis (Vilcek and Havell, 1973; Sehgal and Tamm, 1976; Sehgal et al., 1976). Such observations have led to the suggestion that a post-transcriptional control mechanism, affecting IFNmRNA stability is also involved in controlling IFN production.

i) "Superinduction"of IFN synthesis

A typical "superinduction" protocol involves poly(rI.poly(rC) induction in the presence of cycloheximide. At 4h after induction (when IFNmRNA synthesis is complete) RNA synthesis is irreversibly inhibited by actinomycin D, then at 5h the cycloheximide block is

continues for up to 4 days.

To investigate the phenomenon poly(A)$^+$RNA from poly(rI).poly(rC) induced and "superinduced" cells was extracted at various times after induction. The relative amounts of IFNmRNA in these RNA samples was estimated by microinjection into Xenopus oocytes and assay of translated IFN activity (Sehgal et al., 1977; Cavalieri et al., 1977a). In cells induced by poly(rI).poly(rC), translatable IFNmRNA accumulated to a maximum by around 2-3h after induction then rapidly decayed with an apparent half-life of 18 mins. "Superinduced" cells showed a similar accumulation of IFNmRNA, however its functional stability was increased, showing an apparent half-life of around 68 mins. The increased stability of IFNmRNA was proposed to be the cause of the increased production of IFN.

Although there is some evidence to suggest that "superinduction" can alter the period of transcription and the amounts of IFNmRNA synthesised, the rate of accumulation of IFNmRNA is not altered (Cavelieri et al., 1977). Increased IFNmRNA stability alone is sufficient to explain the increased IFN production, in which case "superinduction" should more properly be called "superproduction" since the effect is on production rather than induction of IFN synthesis.

ii) The post-transcriptional repressor hypothesis

Metabolic inhibiters have been observed to produce similar effects on the synthesis of a number of other inducible proteins, such as steroid induced tyrosine amino transferase. By analogy it was suggested that a McAuslan-Tomkins type translational repressor (McAuslan, 1963; Tomkins et al., 1972) was involved in the inactivation of cytoplasmic IFNmRNA and shut-off of IFN synthesis. All of the evidence is consistent with the hypothesis that a repressor mRNA

and protein, with a short half-life of around 3-4h, is co-ordinately induced with IFN and responsible for inactivating IFNmRNA. Firstly, both protein and RNA synthesis is required for termination of IFN synthesis. Secondly, when RNA and protein synthesis is inhibited during the shut-off of IFN synthesis, the decline in IFNmRNA levels continues at the same rate for 3-4h then proceeds at a slower rate. Thirdly, when RNA synthesis is partially and reversibly inhibited by DRB from the time of induction, increased IFN production occurs after removal of inhibitor despite the fact that IFNmRNA synthesis is reduced (Vilcek and Havell, 1973; Sehgal and Tamm, 1976; Sehgal et al., 1976). It has been argued that the inactivation of IFNmRNA is selective, since its degradation during the shut-off of IFN synthesis is not accompanied by a change in the stability of total poly(A)$^+$mRNA in the cells (Sehgal and Gupta, 1980). Similarly it was shown that increased IFN production in inhibiter-treated cells could not be accounted for by reduced mRNA competition due to the depletion of short half-life mRNA (Sehgal and Tamm, 1976).

Unfortunately "superinduction" has not been demonstrated in virus induced cells. This could be due to an effect of the inhibitors on virus replication or the formation of the proximate inducer. Alternatively, it could be that the induction or regulation of IFN production is different in poly(rI).poly(rC) and in virus induced cells. If this were the case the validity of poly(rI). poly(rC) induced IFN synthesis as a model for studying IFN production is questionable. This point is discussed further in section 4.

To conclude, the "superinduction" of IFN synthesis is consistent with the hypothesis that the synthesis of a post-transcriptional repressor molecule is reduced in inhibitor-treated cells, resulting in increased mRNA stability and a delay in the shut-off of IFN production.

However, direct evidence for the specificity of the repressor will rest on identification of the molecular mechanisms involved in controlling IFNmRNA stability.

Other observations relating to the control of IFN production

Two other phenomena which are relevant to a discussion of the regulation of IFN synthesis involve the effect of IFN itself on IFN production and the responsiveness of cells to induction following the shut-off of IFN production.

i) "Priming"

Treatment of cells with IFN, or "priming", before induction has several effects on the induction and regulation of IFN synthesis. These effects have been characterised mostly in mouse cells and can be summarised as follows. 1) "Primed" cells produce increased amounts of IFN when induced by viruses or poly(rI).poly(rC). 2) "Priming" shortens the lag phase in virus induced cells (but not poly(rI).poly(rC) induced cells). 3) The inducer-cell interaction appears to be affected since "primed" mouse cells no longer require DEAE-dextran treatment to respond to poly(rI).poly(rC). (StewartII 1979). "Priming" develops rapidly, within 1h of treatment with IFN, and does not require protein synthesis. It therefore differs from many other IFN induced effects, such as the antiviral state, which involve new protein synthesis and require several hours to establish.

There is no clear explanations for the "priming" effect, especially as there is conflicting information about the effect on IFNmRNA synthesis. In NDV-induced mouse cells "priming" does not alter the amount of IFNmRNA synthesised or its rate of synthesis and degradation, although both IFNmRNA and IFN appeared 2-4h earlier

than in "unprimed cells" (Abreu et al., 1979; Content et al., 1980). It was therefore suggested that "priming" increased the efficiency of IFNmRNA translation. However increased amounts of IFNmRNA are found in primed-poly(rI).poly(rC)-induced mouse cells (Fujita et al., 1979) and also in primed-poly (rI). poly(rC)-induced human fibroblasts (Sehgal and Gupta, 1980). Although it is tempting to speculate that "priming" has a functional role, the mechanism of its effect on the induction and regulation of IFN synthesis remain unknown.

ii) The hyporesponsive or refractory state.

The induction, production and shut-off of IFN synthesis is followed (in most cells) by a hyporesonsive period, lasting several days, during which time the cells are refractory to a secondary induction of IFN synthesis (Stewart, 1979). It is unlikely that IFN itself is the direct cause since there is no correlation between hyporesponsiveness and the persistance of IFN mediated effects such as the antiviral state. It has been suggested that the hypothetical post-transcriptional repressor which is responsible for IFNmRNA inactivation persists after the shut-off of IFN synthesis and prevents translation of IFN from a secondary induction. Support for this idea will require identification of the repressor and its period of synthesis.

Differential expression of human IFN-α and IFN-β genes

Very little is known about the coordination of IFN-α and IFN-β gene expression. Both types of IFN gene appear to be available for transcription and respond to the same inducer, yet in different cell-types and with different inducers either IFN-α or IFN-β are preferentially expressed. For example, 80-90% of the IFN activity produced by the Namalwa lymphoblastoid cell line was characterised antigenically as IFN-α, the remainder as IFN-β (Havell et al., 1977).

The composition of IFN produced by human fibroblasts varies depending on the inducer. After poly(rI).poly(rC) induction only IFN-β activity can be detected, whereas NDV induction results in a mixture of approximately 80% IFN-β and 20% IFN-α(Hayes et al., 1979). It is likely that this differential expression is controlled at the level of transcription or RNA processing since the IFNmRNA present in these cells qualitatively (and possibly quantitatively) reflects the type of IFN they express. Thus NDV or Sendai virus induced lymphoblastoid cells contain both IFN-α and IFN-β mRNA, determined both by translation and characterisation of IFN activity in oocytes and by nucleic acid hybridization (Cavalieri et al., 1977b; Sagar et al., 1981; Gross et al., 1981; 1982). The quantitation of translated IFN activity showed that the relative proportions of IFN-α and IFN-β mRNA were similar to the IFN-α and IFN-β activity expressed by FS24 cells (Pang et al., 1980) Similarly poly (rI).poly(rC) induced fibroblasts contain only IFN-β mRNA (Cavalieri et al., 1977b; Raj and Pitha, 1981; Sehgal and Sagar, 1980). However, cytoplasmic stability could be involved in regulating IFNmRNA levels.

The obvious implication from these observations is that cell phenotype determines which IFN is predominantly expressed, however the functional significance of this is not known.

d) <u>Expression of human IFN genes in heterologous cells</u>

The introduction of human IFN genes into a heterologous cell environment has provided some interesting information about the induction and regulation of IFN synthesis. Two experimental approaches have been used:

i) Human-mouse somatic cell hybrids have been constructed. The hybrid cells phenotypically resemble the mouse parent because of the preferential loss of human chromosomes and retention of mouse

chromosomes. Provided that human chromosome 9 is retained these hybrid cells can be induced by NDV to produce both human and mouse IFNs which can be distinguished by their target cell specificity. It was found that the control of human IFN production conformed to the dominant mouse parental phenotype. For example the kinetics of IFN production are different in each of the parent cells, however human IFN production in the hybrid cells was regulated with the same kinetics of the mouse parent (Meager et al., 1979b; Graves and Meager 1980). Likewise human IFN production in the hybrid cells was no longer "superinducable". However, only human IFN-β was expressed regardless of whether human fibroblasts (predominantly IFN-β producers) or human leukocytes (predominantly IFN-α producers) were fused with the mouse cells (Meager et al., 1979b; Slate and Ruddle, 1979). The reasons for this are not apparent. However, since the kinetics of IFN production (both human and mouse) were determined by the dominant mouse phenotype, the induction and regulation of IFN production did not primarily involve species-specific mechanisms, although human genes other than the IFN genes could be utilised.

ii) The transfer of cloned human IFN genes into mouse cells will potentially provide more information about the mechanisms of induction and regulation. DNA containing either the human IFN-α1 or IFN-β gene coding and flanking regions has been introduced into mouse L tk^- cells by calcium phosphate coprecipitation (Mantei and Weissmann, 1982; Ohno and Taniguchi, 1982; Hauser et al., 1982). Both of these genes can be induced by NDV and are expressed in parallel with mouse IFN, apparently in agreement with the somatic-cell hybrid results discussed above. The only evidence for expression of the IFN-α1 gene was the presence of low levels of human IFN-α1 transcripts in induced cells and no human IFN-α1 activity could be

detected. Whether this meant that no IFN-∝1 was translated or simply that synthesis was below the limits of detection is not clear. Because of the high cross-reactivity of human IFN-∝ with mouse cells it would be difficult to distinguish small amounts of activity in the presence of mouse IFN. The failure to detect human IFN-∝ synthesis in cell hybrids and transformed cells could therefore be due either to restricted transcription or translation.

Site directed mutagenesis of cloned genomic IFN DNA and expression in surrogate systems such as this will potentially be able to identify those regions of the IFN genes which are involved in induction and regulation of transcription. These experiments have already provided indirect evidence suggesting that induction does in fact increase transcription of the IFN genes rather than increase the processing and stability of constitutively produced transcripts. Transfer of the human IFN-∝1 gene into mouse cells resulted in constitutive expression of low levels of IFN-∝1 transcripts which showed incorrect initiation upstream from the normal mRNA cap site (Mantei and Weissmann 1982). NDV induction resulted in a significant increase in correctly initiated human IFN-∝1 mRNA while the level of incorrectly initiated transcripts remained the same. It was argued that since the level of incorrectly initiated transcripts did not change, IFNmRNA stability was not altered during induction and that increased transcription of the IFN genes produces the correctly initiated mRNA. This would obviously depend on two assumptions; firstly, that correct 5' termini are not involved in determining mRNA processing and stability, and secondly that the correctly and incorrectly initiated constitutive transcripts are produced in the same cells.

e) Summary

It should be clear from the previous sections that studies on

the regulation of IFN gene expression have provided more questions than answers. Although the cloning and characterisation of the human IFN-α and IFN-β genes has increased our understanding of the nature andproperties of this structurally and functionally related family of proteins, considerable gaps remain. The mechanisms controlling the transcription of these IFN genes and the synthesis, stability and translation of their mRNA remain obscure. Expression of the IFN-α and IFN-β genes appears to be regulated at the transcriptional level and control of IFNmRNA stability in the cytoplasm has also been implicated in the shut-off of IFN synthesis.

The availability of an extremely sensitive bioassay and monoclonal antibodies for IFN protein, and also cloned DNA probes, overcomes many of the disadvantages associated with studying the expression of moderately abundant mRNA and protein. No comparable system has yet been described, therefore apart from the intrinsic interest, the expression of IFN genes in cultured human cells is potentially a useful model for studying the expression of eukaryotic genes.

Section 1.3 IFN production by Sendai virus-induced Namalwa cells: a system for studying the control of human IFN-α and IFN-β gene expression

Human IFN-α and IFN-β production, purification and characterisation has essentially involved only three systems: 1) Poly(rI),poly(rC)-induced fibroblasts such as the FS-4 line (Havell and Vilcek, 1972), 2) Sendai virus-induced peripheral blood leukocytes prepared from "buffy coats" (Cantell and Hirvonen, 1977) and 3) Sendai virus-induced lymphoblastoid cells such as the Namalwa cell line (Strander et al., 1975). These systems were chosen either because of their convenience or high yields of IFN, both important considerations for obtaining large quantities of pure IFN for experimental use. Much of the information obtained from these systems has been considered in section 1.2, from which it will be apparent that each has several important differemces. From the point of view of choosing a system to study the regulation of gene expression the following points were considered.

i) IFN-β production in poly (rI).poly(rC) induced and "superinduced" fibroblasts has been well characterised, and provides the basis for most current ideas about the regulation of IFN synthesis. Apart from the questionable validity of using an artificial inducer such as poly(rI).poly(rC), IFN production in other cell-lines, including IFN-α and IFN-β producers, should be investigated in order to expand ideas about the regulation of gene expression.

ii) Human leukocytes could provide an alternative system to study IFN-α production. However they have several disadvantages. Firstly,

they are inconvenient to obtain in large quantities and cannot be readily established in culture. Secondly, they consist of a mixed population of cells. Therefore leukocytes do not provide a homogeneous, reproducable system even though they may be closer to the *in vivo* situation to which all information must eventually be related.

iii) Lymphoblastoid cell lines, such as Namalwa, provide an attractive alternative. They can be grown in culture indefinitely and produce high yields of IFN (both IFN-α and IFN-β). Furthermore, IFN synthesis can be modulated by the use of agents such as butyrate and BrdUrd (see section 1.1). For these reasons Sendai virus-induced Namalwa cells were chosen to study the control of IFN gene expression. The remainder of this section considers some of the characteristics of this system and the question it provides.

Namalwa cells

Namalwa cells are an Epstein-Barr virus (EBV) transformed, human B-lymphoblastoid cell line which was derived from a patient with Burkitt's lymphoma (Klein et al., 1972). The value of Namalwa cells as a source of human IFN was realised when they were shown to produce high levels of IFN after induction by Sendai virus (Strander et al., 1975). Various B-lymphoblastoid cell lines, established either from patients with EBV-related diseases (such as Burkitt's lymphoma and nasopharyngeal carcinoma) or by *in vitro* transformation of leukocytes with EBV, have been examined for IFN production. While most of these cell lines could be induced to produce IFN, the ability to produce high yields of IFN was not a general property (Strander et al., 1975; Christofins et al., 1981).

IFN production in these transformed cells does not appear to be affected by the resident EBV DNA. All EBV transformed cell-lines

express Epstein-Barr nuclear antigen, but the expression of other virus coded products such as early antigen (EA) and virus capsid antigen (VCA) is normally répressed. These markers can be induced in some latently infected B-lymphoblastoid lines by halogenated pyrimidines, butyrate and the tumour promoter phorbol myristate acetate (Saemundsen et al., 1980; Klein and Vilcek, 1980). No relationship was found between the induction of EBV markers and the production of IFN. In any case, Namalwa cells only carry 2 incomplete copies of the EBV genome, and neither EA or VCA can be detected before or after treatment with butyrate or BrdUrd (Pritchett et al., 1976; Tovey et al., 1977; Klein and Vilcek, 1980).

Namalwa and several other lymphoblastoid cell-lines have been reported to produce IFN constitutively when cultured at high densities of around 10^7 cells/ml (Adams et al., 1975; Tovey et al., 1977. see also section 1.2). Other groups have failed to detect constitutive IFN synthesis in exponentially growing Namalwa cells (Pickering et al., 1980; Rossier and Weideli, 1982). Whether this reflects differences in culture conditions or drift in the properties of different Namalwa stocks is not clear. However it is clear that normal regulatory mechanisms are functional, since virus induction results in a transient increase in IFN production in all Namalwa cells.

Sendai virus

Several viruses have been shown to induce IFN synthesis in Namalwa cells, but paramyxoviruses such as Sendai virus are particularly effective (Strander et al., 1975. see also section 1.2). Sendai virus is a non-segmented, negative-stranded RNA virus. Its 5×10^6 molecular weight genome codes for 5 structural polypeptides (Scheid and Choppin, 1974) with the following functions. 1) The virus RNA polymerase complex (P), 2) the haemagglutination/

neuraminidase glycoprotein (HN), 3) the precursor fusion glycoprotein Fo which is processed to(F), 4) the nucleocapsid subunit (NP) and 5) the membrane protein (M). HN and F are both associated with the surface of the virion and are involved in binding and entry into the host cell.

A characteristic feature of Sendai virus replication is the generation of defective interfering (DI) particles which contain incomplete genomes. Preparations of chicken egg-grown Sendai virus, which have a high proportion of DI particles, are routinely used to induce IFN production. It has been found that the effectiveness of Sendai virus preparations as IFN inducers in Namalwa cells depends on the ratio of DI to infective particles (Johnston, 1981). Standard virus alone was a poor inducer and DI particles were ineffective. Therefore DI particles are responsible for the effectiveness of Sendai virus as an IFN inducer, possibly by causing the accumulation of replicative intermediate which provide the putative double-stranded RNA inducer molecule (see section 1.2).

The control of IFN production in Namalwa cells

The kinetics of IFN production in Sendai induced Namalwa cells have already been well characterised. IFN production is first detected 2-3h after induction and is complete by around 14-16h (Zoon et al., 1978; Baker et al., 1979; Morser et al., 1979; Johnston, 1980). IFNmRNA measured by translation of IFN activity from RNA microinjected into Xenopus oocytes, is first detectable in cells 2-3h after induction, rapidly accumulates to a maximum by around 9h then declines to undetectable levels by around 15h (Morser et al., 1979; 1980). IFN production in Namalwa cells therefore resembles that found in poly(rI).poly(rC) induced fibroblasts (see section 1.2). In both cases IFNmRNA is undetectable in non-induced cells, and induction

causes a rapid increase in IFNmRNA and IFN synthesis which is followed shortly by the inactivation of IFNmRNA and shut-off of IFN production.

However, there are several differences which prevent a direct comparison between the two systems. The timing and type of IFN induced is different in each cell-type. In addition Namalwa cells respond poorly to poly(rI).poly(rC) inductions, little or no IFN being produced (Vervliet - Volckaert et al., 1980; Johnston, 1980). Only one report has indicated that poly(rI).poly(rC) can effectively induce IFN synthesis in Namalwa cells (Rossier and Weideli, 1982). The reasons for the failure to induce IFN are not known, however differences in the binding, release and degradation of poly(rI).poly(rC) suggest that its interaction with lymphoblastoid and fibroblast cells is different (Vervliet - Vockaert et al., 1980).

In addition, IFN production by virus-induced Namalwa cells cannot be increased by "superinduction" schedules analogous to those described for poly(rI).poly(rC) induced fibroblasts (Zoon et al., 1978; Morser et al., 1979). To what extent this is due to cell-type or inducer remains to be established (see section 1.2).

Although Namalwa cells cannot be "superinduced", IFN production can be increased by treating cells with a variety of agents which are known to affect specialised protein synthesis in a number of cell-lines. Agents which increase IFN production include butyrate (Adolf and Swetly, 1979a; Johnston, 1980), dimethylsulphoxide (Adolf and Swetly, 1979a), 5'-bromodeoxyuridine or BrdUrd (Tovey et al., 1977), glucocorticoids (Adolf and Swetly, 1979b) and the tumour promoter phorbolmyristate acetate (Adolf and Swetly, 1980). Two of these agents, butyrate and BrdUrd (see section 1.1), have been used to investigate the control of IFN production in Namalwa cells.

Modulation of IFN production by butyrate and BrdUrd

Treatment of Namalwa cells with butyrate before induction with

Sendai virus has been reported to increase IFN production by up to 30-fold (Adolf and Swetly., 1979; Johnston, 1980) The effect is time and concentration dependant,. reaching a maximum when cells are induced after 48h of treatment with 1mM butyrate (Baker et al., 1980). Treatment also causes many of the other effects described in other cultures, including inhibition of DNA synthesis and cell division and hyperacetylation of histones H3 and H4 (Baker et al., 1980). In addition a partial (60-80%) inhibition of RNA and protein synthesis is observed. No changes in cell morphology occur, but cells become smaller and are arrested in the G1 phase of the cell cycle. All of these effects are fully reversed within 48h of removal of butyrate. The increase in IFN production cannot be caused directly by the changes in DNA synthesis, cell cycle progression and histone acetylation since these occur more rapidly and are complete after only 24-36h of treatment with butyrate.

A similar (up to 32-fold) increase in IFN production has been reported when Namalwa cells are treated with BrdUrd before induction (Tovey et al., 1977). Again the increase is dose-dependant and is maximum after 48-72h of treatment with BrdUrd at 25ug/ml (Baker et al., 1979). At this concentration BrdUrd inhibits cell growth but this can be prevented by adding deoxycytidine (which prevents the cytotoxic effects of BrdUrd) without affecting the stimulation of IFN production. However the increase in IFN production is reduced by the addition of thymidine which also reduces the incorporation of BrdUrd into cellular DNA. The effect of BrdUrd on IFN synthesis therefore correlates with its incorporation into DNA.

These agents have a varied effect on IFN production in other cell-types. For example butyrate increases IFN production in monkey V3 cells but not in human diploid fibroblasts (Johnston, 1980;

Baker et al., 1980). Similarly BrdUrd increases IFN in human diploid fibroblasts but not in SV-40 transformed human fibroblasts. These results indicated that the effects of butyrate and BrdUrd on cellular function and chromatin structure in these cells were not sufficient to affect IFN production.

The significance of all these effects of BrdUrd and butyrate in relation to the control of IFN production is not known. However, recently it was found that the increase in IFN production caused by butyrate was accompanied by increased synthesis of IFNmRNA (Morser et al., 1980). Therefore it appeared that for butyrate at least, the effect on IFN production was occurring at the level of mRNA transcription or processing.

The aims of this investigation

There is still a great deal of information to be obtained before the control of IFN gene expression can be descibed in detail. Many aspects of IFN production in virus induced Namalwa cells, and cultured cells in general, remain poorly defined. With the intention of characterising IFN gene expression in Sendai induced Namalwa cells, the following aspects were considered.

The control of IFN production in poly(rI).poly(rC) induced human fibroblasts has been suggested to occur both at the transcriptional and post-transcriptional level, on the basis of data obtained using metabolic inhibiters (see section 1.2). Similar data on the control of IFN production in Namalwa cells is not available. One section of this thesis investigates the role of IFNmRNA stability in control of IFN production in Namalwa cells and examines the relevance of the post-transcriptional repressor model described in section 1.2.
It has generally been assumed that IFN synthesis is controlled by the availability offunctional IFNmRNA in the cytoplasm. However,

the relationship between amounts of IFNmRNA present in the cells and the amount of IFN produced has received little attention in the literature. This relationship is considered here in order to determine how IFN synthesis is quantitatively controlled.

In addition, IFN gene expression in Namalwa cells has been investigated by characterising the IFNmRNAs which are present after Sendai virus induction. The IFNmRNAs have been studied both by translation in Xenopus oocytes and by hybridization with cloned IFNcDNA probes.

Finally, the quantitative and qualitative effects of butyrate and BrdUrd treatments on IFNmRNA and IFN synthesis are described. The effects of these treatments on IFN gene expression are compared with their effects on gene expression in general by characterising the changes they cause in the synthesis of other proteins in Namalwa cells.

SECTION 2. MATERIALS AND METHODS.

a) Reagents and materials.

All general reagents were obtained either from BDH Chemicals Limited, Poole, England; Fisons Scientific Apparatus, Loughborough, England; Sigma Chemical Company, London. The named reagents and materials below were obtained from the following suppliers:

a - Aderman and Company Limited, Central Avenue, East Mosely, Surrey, England.

BCL - Boehringer Corporation (London) Limited, England.

BRL - Bethesda Research Laboratories Incorporated, Cambridge, England.

b - Collaborative Research Incorporated, Waltham, USA.

c - Eastman-Kodak Company, Rochester, USA.

d -Fluorochem Limited, Dinting Vale Trading Estate, Dinting Lane, Glossop, England.

e - Lancaster Synthesis Limited, Eastgate, White Lund, Morecambe, England.

f - Pharmacia Fine Chemicals,Uppsala, Sweden.

Reagents and materials, listed in alphabetical order, with supplier as indicated above.

acrylamide - c
Actinomycin D - Sigma
N,N'- methylene-bis acrylamide - c
bovine serum albumen - Sigma
5'-bromodeoxyuridine - Sigma
butyrate - BDH
Caesium chloride - BRL
chloramphenicol - Sigma

cyclohexemide - Sigma
DE 52, Whatman - Fisons
DNA polymerase 1, (from E.coli) - BRL
ficoll, Whatman - Fisons
GF-C, glass fibre filters, Whatman - Fisons
guanidinium isothiocyanate - d
methylmercury hydroxide - e
nitrocellulose, 0.45um, Schleicher and Schull - a
oligo (dT) cellulose - b
polyvinylpyrrolidone - Sigma
PPO - BDH
proteinase K - BCL
puromycin - Sigma
RNase A - BCL
RNase T1 - BCL
tetracyclin - Sigma

Radioactively labelled compounds were obtained from The Radiochemical Centre, Amersham, England.

deoxyguanosine 5'-[α-^{32}P] triphosphate.2,000-3,000 Ci/mMol
deoxycytidine 5'-[α-^{32}P] triphosphate.2,000-3,000 Ci/mMol
L-[^{35}S]methionine. >600Ci/mMol
[5,^{3}H]uridine. 27Ci/mMol

Media

Medium RPMI 1640 , GMEM, foetal calf serum and newborn calf serum were all obtained from Flow Laboratories, Irvine, Scotland.

Medium RPMI 1640 was buffered with 20mM Hepes to pH6.9. GMEM was buffered with bicarbonate. All media were supplemented with 60ug/ml penicillin and 100ug/ml strptomycin (Glaxo) before use.

Buffers and solutions.

Barth-X medium (for oocyte incubation) was made up in two parts and stored as a 25X concentrated stock at -20°.

Barth-X	25X (A)	NaCl 2.2M
		KCl 25mM
		Na bicarbonate 600mM
		tris-HCl pH7.35 375mM
	25X (B)	$Ca(NO_3)_2$ 8.25mM
		$CaCl_2$ 10.25mM
		$MgSO_4$ 20.5mM

SSC	20X	NaCl 3M
		Na citrate 0.3M

Denhardt's solution		Ficoll 0.02%
		polyvinylpyrrolidone 0.02%
		bovine serum albumen 0.02%

Borate buffer (for methylmercury-agarose gels)

10X	borate 61.8g	
	Na tetraborate 38.1g	
	Na_2SO_4 28.4g	
	EDTA 7.4g	made up to 2l, pH8.2 with NaOH

Glycine-OH buffer (for agarose gel electrophoresis of DNA)

NaOH, 1M	15ml	
glycine	15g	per l

Cells and viruses.

The Namalwa cells used in these experiments were provided by Wellcome Research Laboratories, Beckenham, England. A separate stock of Namalwa cells (see below) were obtained from Dr.P.Swetly, Ernst Boehringer Institute, Vienna, Austria,for comparison with the stocks used in these experiments.

MDBK (Malin Darby Bovine Kidney cells) and EBTr (Bovine tracheal cells) were a gift from Dr.J.Vilcek, New York Medical Centre, NY, USA.

HFF cells (Human Foreskin Fibroblasts) were a gift from Dr.T. Merigan, Stanford University, Ca, USA.

Sendai virus seed stock (for growth of inducing virus stocks) was provided by Wellcome Research Laboratories, Beckenham, England. Purified normal Sendai virus was a gift from Dr.M.Johnston, Wellcome Research Laboratories.

Characterisation of Namalwa cells.

The characteristics of the Namalwa cells used in these experiments were compared with those of Namalwa cells obtained from an independant source (see above), in order to confirm their identity.

A karyotype analysis was carried out on both cell lines using unbanded, Giemsa stained chromosomes. Modal numbers of 46 for the Wellcome stock, and 45 for the stock from Dr.P.Swetly were obtained from 15 spreads, by camera lucida (Dr.J.Brown, personal communication). No gross differences were observed, and this result agrees with previously published variation between sub-populations of Namalwa cells (Shade et al.,1980).

A direct HLA typing was also carried out on both stocks of Namalwa. The results showed that the cells behaved identically, both being A3, B7, BW21, and DR4 (L. Kennedy, Dr.L.Jones and Dr.J.Morser, personal communication). This result was confirmed by adsorption of sera specific for the polymorphic determinants with 3 x 10^8 cells/ml, and testing in a cytotoxicity assay using fluorescain diacetate and ethidium bromide. These results therefore confirmed that the cells used in these experiments were in fact Namalwa cells.

Characterisation of Namalwa cell IFN.

The IFN produced by the Namalwa cells used in these experiments was characterised by Rob King and Trevor Whittall. Namalwa cells have been reported to produce both IFN-α and IFN-β , from 10-15% of the IFN activity being neutralized by antisera raised to IFN-β (see section

1.2). However neutralization of the IFN produced by the Namalwa cells used in these experiments indicated that a much lower proportion of IFN-β was being produced. Because of the difficulties associated with detecting small proportions of IFN-β (antisera are raised to IFN-β preparations which are contaminated with trace amounts of IFN-α and are therefore cross reactive to a small extent) , an antibody affinity column containing the monoclonal antibody NK2 was used to reduce the high IFN-α background. NK2 binds to the majority of IFN-α species (see below) therefore any IFN-β present would be enriched in the unbound fraction from the column.

Approximately 75% of the IFN from Wellcome Namalwa cells was retained by the column. The unbound fraction was neutralized by anti-IFN-α serum (IFN-α species not retained by NK2), however no neutralization was detected using anti-IFN-β serum (see Table 2). IFN-α has a high cross-species activity (see section1.2), while IFN-β activity is virtually undetectable on cells of another species. The ratio of activity of the unbound fraction in bovine compared to human cells was higher than the starting material (data not shown), again suggesting that it was not IFN-β . Therefore the Namalwa cells used in these experiments did not produce detectable IFN-β activity.

By comparison, the Namalwa cells provided by Dr.P.Swetly produced IFN activity which was readily detected as a mixture of both IFN-α and IFN-β , Therefore the failure to detect IFN-β in the IFN produced by Wellcome Namalwa cells was not due to the methods used in analysis. (see Table 2)

Characterisation of IFNcDNAs.

Information relating to the IFNcDNAs used to characterise IFNmRNA in section 3.3 was provided by Dr.P.Boseley, Dr.P.Slocombe and Dr.A. Easton. The nucleotide sequence of the cDNA inserts contained in the

Table 2. Characterisation of Namalwa IFN.

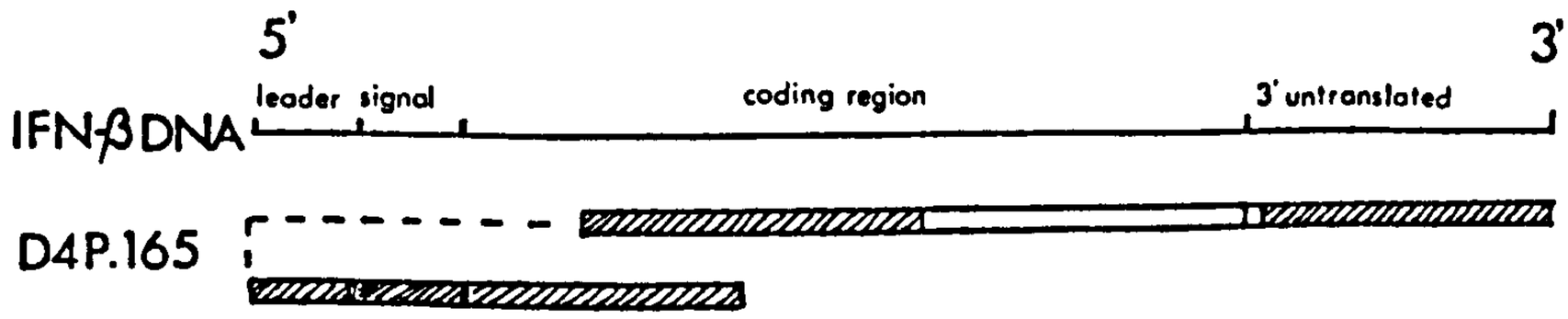
		IFN (log_{10}units/ml)	% neutralized
Namalwa-A IFN		5.40	
unbound		4.70	
unbound+anti-IFN-α	serum	2.80	99
unbound+anti-IFN-β	serum	4.75	0
Namalwa-B IFN		4.50	
unbound		3.70	
unbound+anti-IFN-α	serum	2.20	97
unbound+anti-IFN-β	serum	2.45	44

The IFN activity produced by Namalwa cells was characterised using affinity chromatography and neutralization with anti-IFN sera as described in the text. Namalwa-A are the cells provided by Wellcome Research Laboratories. Namalwa-B were provided by Dr.P.Swetly. Unbound refers to the fraction of IFN not bound by an NK2-sepharose (monoclonal antibody affinity)column. (% neutralized refers to the reduction in activity of unbound fraction)

hybrid plasmids N5H8 and D4P.165 have been obtained and compared with published data for the IFN-α and IFN-β genes. N5H8 was found to contain a complete IFN-α2 coding and 3' untranslated sequence together with part of the protein signal sequence coding region (Slocombe et al.,1982). Fig.3. shows a comparison of the cDNA sequence contained in N5H8 with the published sequence of Goeddell et al.(1980) for IFN-α2 (or LeIFN A). Identical areas are indicated by lines (with arrows in the direction of reading) beneath the IFN-α2 sequence, and differences in nucleotides are noted. The N5H8-Msp fragment also contains approximately 240 base pairs of plasmid DNA, therefore the size of the IFN-α2 cDNA probe derived from N5H8 is approximately 1.185Kb.

D4P.165 was found to contain a complete IFN-β cDNA sequence, however the 5' regions of the insert contained rearranged sequence derived from the non-coding strand. Fig.4a. shows sequenced regions of the cDNA and the nature of the rearrangement which has probably arisen from a strand switch during the preparation of the cDNA.

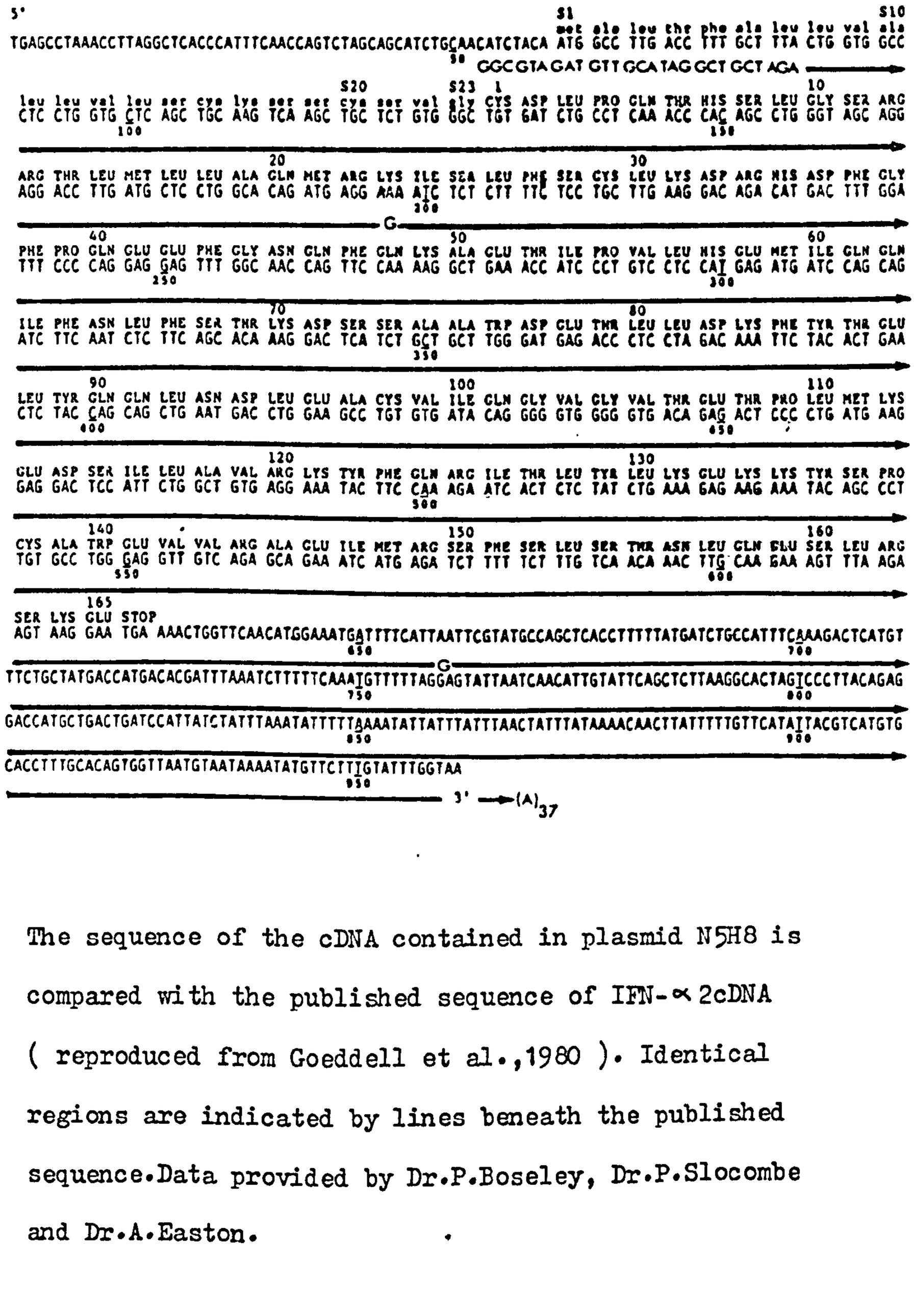
Fig.4a. The rearranged IFN-β cDNA derived from D4P.165.



Sequenced regions are indicated by hatched areas. The region of sequence from the non-coding strand is joined to the 5' end of the coding strand as indicated. Arrows show the direction of reading.

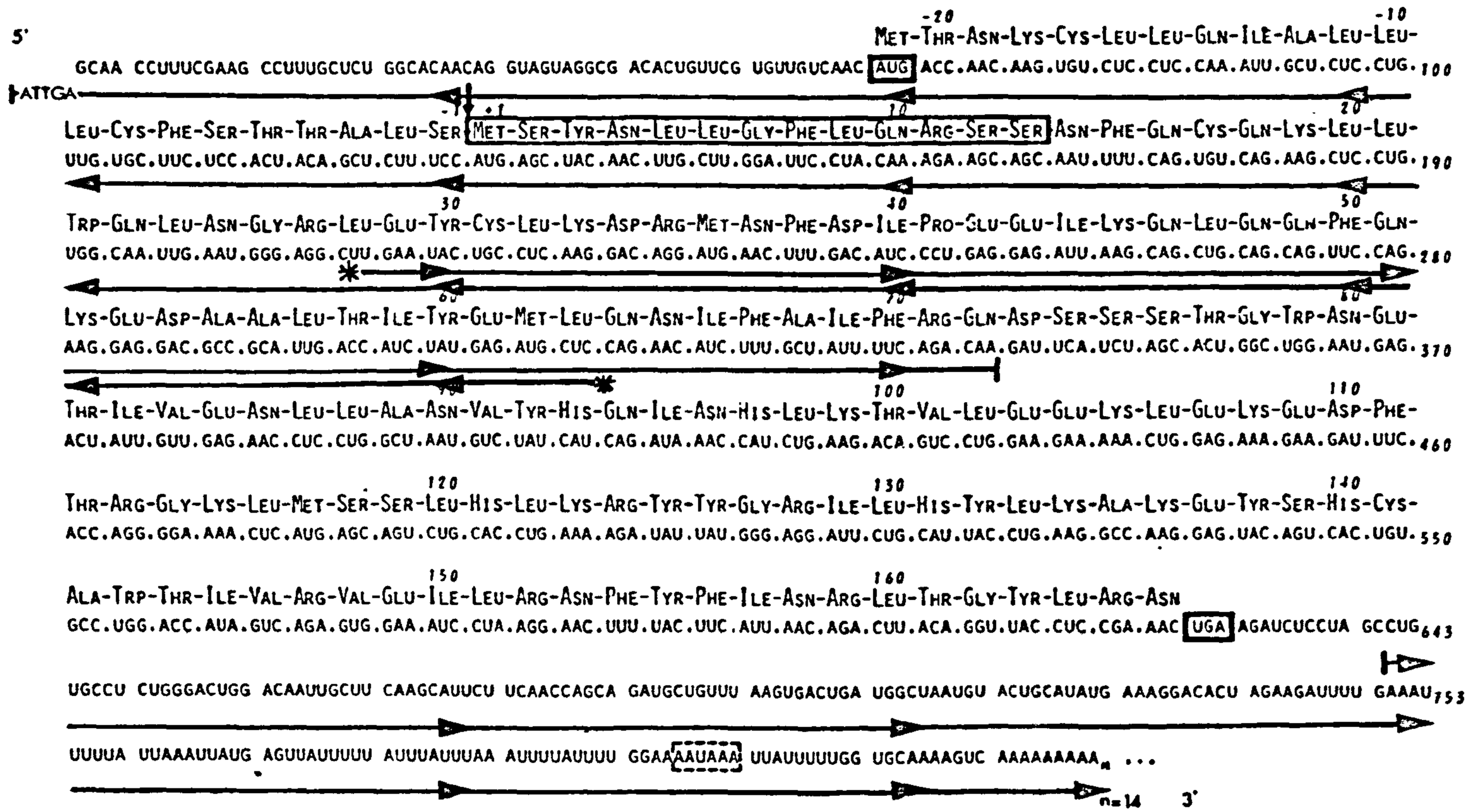
Fig.4b. shows a comparison of the sequenced regions of the cDNA contained in D4P.165 with the published sequence of IFN-β cDNA (Derynck et al.,1980). Sequenced regions are indicated by lines (with

Fig.3. The nucleotide sequence of the IFN-α2cDNA probe.



The sequence of the cDNA contained in plasmid N5H8 is compared with the published sequence of IFN-α2cDNA (reproduced from Goeddell et al.,1980). Identical regions are indicated by lines beneath the published sequence.Data provided by Dr.P.Boseley, Dr.P.Slocombe and Dr.A.Easton.

Fig.4b. The nucleotide sequence of the IFN-β cDNA probe.



The sequence of the cDNA contained in plasmid D4P.165 is compared with the published sequence of IFN-β cDNA (reproduced from Derynck et al.,1980). The sequenced regions are indicated by lines beneath the published sequence . The rearranged region, derived from the non-coding strand is joined to the 5' coding strand at the point indicated by the asterisk. Data provided by Dr.P.Boseley, Dr.P.Slocombe and Dr.A.Easton.

arrows in the direction of reading)beneath the published IFN-β sequence. The D4P.165-Msp fragment contained approximately 240 base pairs of plasmid DNA, therefore the size of the IFN-β cDNA probe used in section 3.3 was approximately 1.214Kb.

b) Methods.

Cell culture.

Namalwa cells were grown in suspension culture at 37° in medium RPMI 1640 supplemented with 10% newborn calf serum, either in 2.5 l roller bottles or medical flats for small volume cultures. The cells were passaged routinely every 2days or as soon as the cell density exceeded 10^6 cells/ml.

The cells used for assay of IFN activity were cultured in 150cm^2 plastic tissue culture bottles or 1l flow bottles in the following media. EBTr,MDBK and HFF were grown in GMEM supplemented with 10% newborn calf serum. GM 2767 were grown in GMEM supplemented with 10% foetal calf serum.

Butyrate and BrdUrd treatment of cells.

Unless otherwise stated, cells were incubated at a cell density of 10^6 cells/ml in maintenance medium* containing 0.8 mM butyrate or at $5x10^5$ cells/ml in maintenance medium containing 25 ug/ml BrdUrd for 48h before induction (Baker et al., 1979;1980).

* RPMI 1640 supplimented with 2% newborn calf serum.

Induction of IFN synthesis.

Treated and untreated cells were resuspended in fresh maintenance medium at a cell density of $2x10^6$ cells/ml and induced using 100 haemagglutinating units of Sendai virus per 10^6 cells (Baker et al., 1979). Chicken egg grown virus was routinely used as this contained DI particles and was found to be an efficient inducer (Johnston,1981). IFN was harvested at the times indicated in each experiment by pelleting the cells at 2,500xg for 10 mins. The supernatant was adjusted to pH2 using HCl in order to inactivate remaining virus before assaying IFN activity.

Intracellular IFN levels were estimated by measuring the activity

in supernatants prepared by lysing cells in HCl and pelleting cell debris as described by Morser et al.,1980.

IFN assay.

IFN activity was assayed by the inhibition of virus nucleic acid synthesis method (Atherton and Burke, 1975). Briefly, IFN samples were added to cells and incubated for 12-18h. Cells were then challenged with SFV and the replication of virus measured by the incorporation of ^{3}H-uridine into viral nucleic acid in the presence of Actinomycin D. 1 unit of IFN is defined as the amount of IFN causing a 50% reduction in viral nucleic acid synthesis. All IFN titres are expressed in international units based on the human leukocyte interferon standard (69/19). The sensitivity of the various cell lines used to assay IFN is different. In the assays used 1 international unit was found to be equivalent to 10units in EBTr cells, 4 units in MDBK cells, 0.5 units in HFF cells and 10 units in GM 2767 cells.

Immunoradiometric assay of IFN was performed using the monoclonal antibody NK2 (Secher and Burke,1980). The procedure used was that described by Secher (1981), and the reagents were kindly provided by Dr. D. Secher. Briefly, the IFN present in cell supernatants was adsorbed to a sheep anti-IFN-α serum immobilised on polystyrene beads. ^{125}I-labelled NK2 monoclonal antibody was then bound to the adsorbed IFN and counted. The assay was linear for samples ranging from 200-10,000 units/ml.

Affinity chromatography, using the monoclonal antibody, NK2, bound to Sepharose was performed as described by Secher and Burke (1980), except that washing with ethanediol was not used. Using these procedures approximately 75% of the IFN-α protein in Namalwa IFN is bound by the affinity column (Allen et al.,1982).

Preparation of RNA.

Total cellular RNA was extracted using the guanidinium isothiocyanate technique (Morser et al.,1979). Cells were pelleted, washed twice in cold PBS and lysed in 4M guanidinium isothiocyanate, 1M 2-mercaptoethanol, 20mM sodium acetate pH5.0. The lysate was then layered over a 5.7M CsCl cushion and centrifuged overnight at 137,000 x g. The RNA pellet was dissolved in 5mM tris-HCl pH7.5 and extracted 3 times with water-saturated butanol:chloroform (1:4) then ethanol precipitated. The RNA was washed 3 times with 80% ethanol before use.

Cytoplasmic RNA was prepared by phenol:chloroform extraction of post-mitochondrial supernatants as follows. Cells were pelleted and washed twice with cold PBS. Cells were then swollen in a hypotonic buffer containing 10mM KCl, 5mM Mg acetate, 20mM tris-HCl pH7.5 at 4^{o} for 5min. The cells were then lysed using 0.5% Nonidet P-40 in a Dounce homogenizer (10 strokes). The homogenate was centrifuged at 10,000 x g for 10min and the supernatant was treated with 0.5% SDS and 100ug/ml proteinase K at room temperature for 15min. NaCl was then added to 300mM and the supernatant extracted 3-4 times with phenol:chloroform (1:1) and 3 times with chloroform. The RNA was then precipitated with 2.5 volumes of ethanol. The precipitated RNA was washed 3 times with 80% ethanol before use.

RNA was measured by determining the A_{260} of solutions in 5mM tris-HCl. Samples were stored at -20^{o} under ethanol.

Selection of poly(A)$^{+}$ RNA.

Poly(A)$^{+}$ RNA was selected by oligo(dT)cellulose chromatography. Total or cytoplasmic RNA at a concentration of 1mg/ml or less was loaded onto an oligo(dT) cellulose column in 10mM tris-HCl pH7.5, 350mM LiCl, 1mM EDTA, 0.1% SDS, at 4^{o}. The column was then washed thoroughly with several bed volumes of the loading buffer, at 4^{o}, then the bound RNA was eluted with 10mM tris-HCl pH7.5, 1mM EDTA, 0.1% SDS at 37^{o}. The

bound RNA was reloaded onto the column and eluted for a second time, using the above conditions, then ethanol precipitated.

Preparation of polysomes.

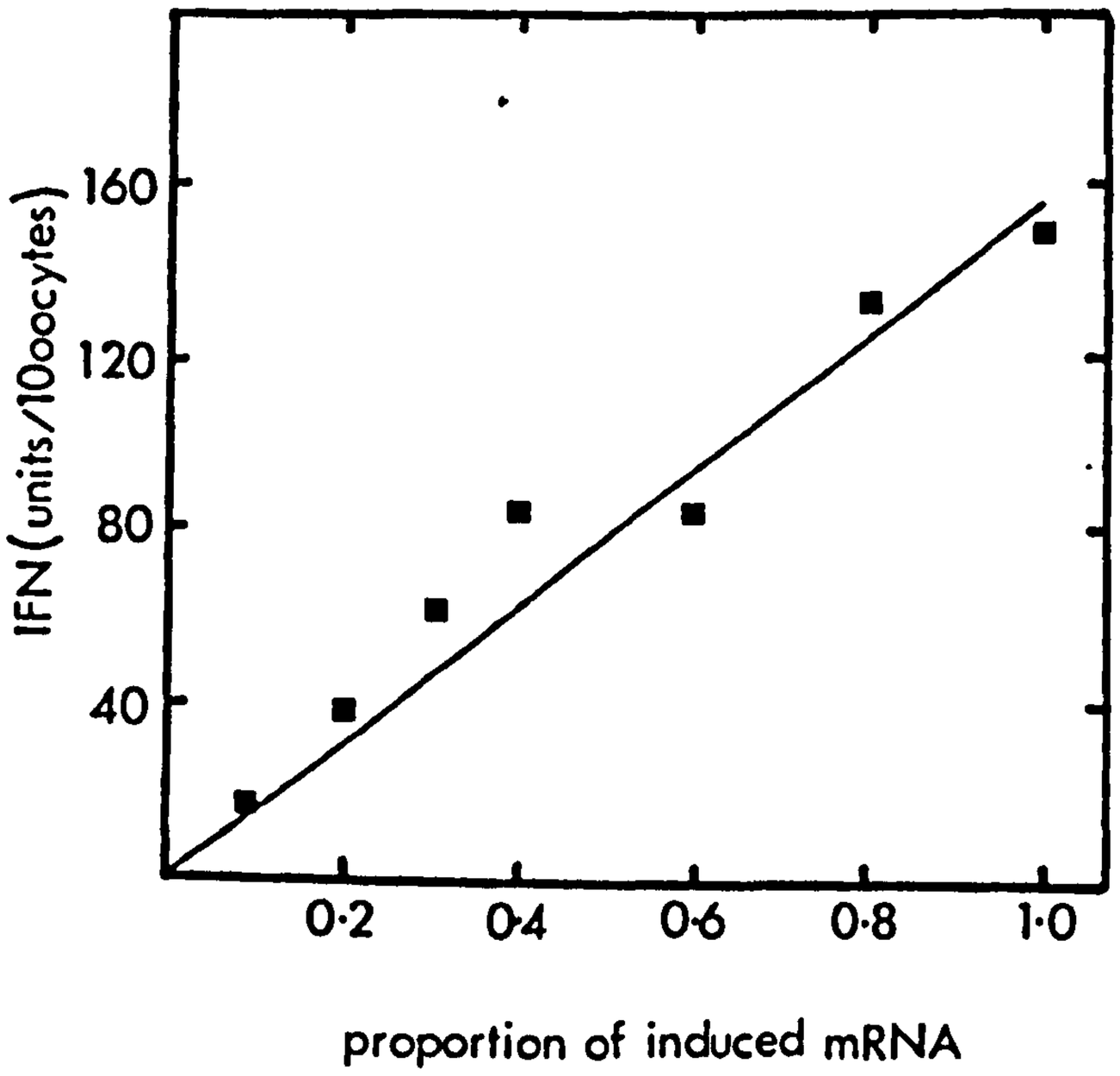
Polysomes were prepared by the method described by Morser and Shuttleworth (1981). Briefly, post-mitochondrial supernatants prepared as for cytoplasmic RNA extraction except that 0.25 volumes of 1.25M sucrose, 460mM KCl were added to the homogenate before centrifugation. The supernatant was treated with 0.1% sodium deoxycholate then centrifuged through a discontinuous sucrose gradient consisting of equal volumes of 1M and 1.8M sucrose in 100mM KCl, 5mM Mg acetate, 20mM tris-HCl pH 7.5. Centrifugation was for 3h at 140,000 x g. RNA was extracted from the polysome pellet by dissolving the pellet in guanidinium isothiocyanate and centrifugation through CsCl as described above.

Microinjection of oocytes.

Total and poly(A)$^+$ RNA was microinjected into oocytes from mature Xenopus laevis frogs as described by Colman and Morser (1979), for the purpose of estimating the relative amounts of IFNmRNA present. Briefly, each RNA sample was assayed by microinjecting batches of 10oocytes with 30nl of RNA solution (at 1-5 mg/ml in distilled water) per oocyte. After 24 h incubation at 21^{o} in 300ul of Barth-X solution, the IFN secreted by the oocytes was assayed.

The amount of IFN secreted by the oocytes was found to be directly proportional to the amount of IFNmRNA injected over the range of RNA concentrations used in the assay. Fig.5. shows the amount of IFN secreted by oocytes which had been microinjected with various concentrations of total RNA extracted from induced cells. Total RNA from non-induced cells was used to maintain a constant input of RNA.

Fig.5. Calibration of the oocyte translation assay for IFNmRNA.



Methylmercury-agarose gel electrophoresis.

Poly(A)$^+$RNA was fractionated by electrophoresis through vertical methylmercury-agarose gels. 10-20ug of RNA was first denatured in 20mM methylmercury hydroxide for 10min at room temperature. 1.5% agarose gels containing 5mM methylmercury hydroxide were prepared in borate buffer. Samples were loaded onto gels in borate buffer containing 3% Ficoll and 0.005%bromophenol blue, and electrophoresed at 50V for 12-15h. All operations were performed in a fume hood (methylmercury is highly toxic).

After electrophoresis the gels were soaked for 30min in either 0.5M ammonium acetate before transferring the RNA to nitrocellulose filters, or 100mM 2-mercaptoethanol, 10mMtris-HCl pH7.5 before extracting RNA for translation in oocytes (see below).

RNA was extracted from the gels by manually slicing tracks into 2mm segments. The excised gel slices were then homogenized,using a sterile glass rod, in 5 volumes of 5mM tris-HCl pH7.5 containing 5ug/ml yeast RNA carrier. The RNA was then allowed to diffuse out of the agarose at 4^o overnight. After pelleting the agarose the RNA solution was extracted

twice with water-saturated butanol:chloroform (1:4) and then ethanol precipitated. The RNA pellet was washed three times with 80% ethanol before use.

The size of RNA fractionated by agarose gel electrophoresis was estimated using ^{32}P-labelled λ-Hind III/ Eco R1 restriction fragments (kindly provided by Dr.P.Turner). These were electrophoresed in adjacent tracks, blotted onto nitrocellulose and visualised by autoradiography.

Preparation of IFNcDNA probes.

IFN-α2 and IFN-β cDNAs were prepared from the recombinant plasmids N5H8 and D4P.165 respectively. Both plasmids were derived from pAT153 by insertion of IFNcDNA into the plasmid tetracyclin resistance gene (Slocombe et al.,1982 ; Dr.P.Boseley, Dr.P.Slocombe, and Dr.A.Easton personal communication).

a) preparation of plasmid DNA

: E.coli (HP101) containing the plasmids N5H8 or D4P.165 were grown using standard procedures, in L-broth containing 100ug/ml Ampicillin ., to a density of 0.8 (at 590nm). Plasmid DNA was amplified overnight in the presence of 100ug/ml chloramphenicol. Cleared lysates were then prepared from cell pellets by the following procedure. Cells were resuspended in 25% sucrose, 50mM tris-HCl pH8.0, and treated with 1.6mg/ml lysozyme for 5min on ice. The cells were then treated with 60mM EDTA pH8.0 for 5min on ice and an equal volume of 0.1% Triton-X 100, 62mM EDTA, 5mM tris-HCl pH 8.0 added. The cells were shaken vigorously. After centrifugation at 20,000 rpm, 1h, 4°, the supernatant was extracted with phenol:chloroform (1:1) several times and dialysed extensively against 10mM tris-HCl pH8.0, 1mM EDTA. DNA was then ethanol precipitated.

Precipitated DNA was washed with 80% ethanol, resuspended in 10mM tris-HCl pH7.5, 1mM EDTA, 300mM NaCl and digested with T1 RNase and

RNase A (3ul and 50ul/10ml respectively) for 1h at 37°. After phenol: chloroform extraction the DNA was then reprecipitated.

DNA was then resuspended in 0.1 x SSC, added to 20ml of 0.1 x SSC containing 26g of CsCl and 0.6mg/ml ethidium bromide and centrifuged at 50,000rpm, for 18h at 23° in a Beckman VTi 50 rotor using a Beckman L8 centrifuge. Banded plasmid DNA was collected and extracted 7 times with isoamyl alcohol to remove ethidium bromide. The DNA was then ethanol precipitated.

b)Preparation of cDNA inserts.

IFNcDNA was purified from plasmid DNA by restriction enzyme digestion and agarose gel electrophoresis. Plasmid DNA was digested with Msp 1 and the cDNA frgment fractionated by electrophoresis through 1.5% agarose gels prepared and run in glycine-OH buffer. Gels were stained with ethidium bromide and the appropriate band excised. DNA was extracted from the agarose (by homogenisation and diffusion overnight, into 10mM tris-HCL pH 7.5, 1mM EDTA) and purified by DE52 chromatography.

c) ^{32}P-labelling of cDNA probes.

IFNcDNAs were labelled by nick-translation with E.coli DNA polymerase 1,using ^{32}P-labelled deoxynucleotide triphosphates. Labelled DNA was separated from unincorporated deoxynucleotide triphosphates by sephadex G-50 chromatography and used without further purification. The specific activity of these probes was usually in the order of $2x10^8$cpm/ug of input DNA.

Dot-blot hybridization with RNA.

Poly(A)$^+$ and total RNA was analysed by dot-blot hybridization essentially as described by Thomas, 1980. 5ug of poly(A)$^+$ or 20ug of total RNA dissolved in 5mM tris-HCl pH7.5, were loaded onto nitrocellulose filter discs which had been soaked in 10 x SSC and dried. The filters were then baked at 80° in a vacuum oven, then prehybridized at 37°for at least 5h in 50% deionized formamide, 3 x SSC, 1 x Denhardt's solution, 20mM sodium phosphate pH 6.5 and 50ug/ml denatured salmon sperm DNA. Hybridization was performed in the same solution for 48h using approximately 10^7cpm of denatured nick-translated IFNcDNA probe. In some hybridizations using IFN-α 2cDNA the temperature of hybridization was reduced to 20^0 for the reasons indicated in section 3.

Following hybridization the filters were washed for 1h at room temperature in 3 x SSC then for 15min at 60° in 0.1 x SSC then counted by Cerenkov emmission.

Gel-transfer hybridization.

RNA was transferred from methylmercury-agarose gels by blotting onto nitrocellulose filters. After soaking in 0.5M ammonium acetate the RNA was transferred to nitrocellulose filter sheets essentially as described by Southern, using 10 x SSC. Transfer (or retention)of RNA was found to be increased by soaking the gels in 20 x SSC before blotting. Following transfer of RNA the filters were dried, baked and hybridized as for dot-blots (see above).

Hybridized filters were autoradiographed using a Dupont Cronex intensifying screen at -70° for 1-5days.

Labelling of cells with ^{3}H-uridine and ^{35}S-methionine.

Incorporation of ^{3}H-uridine and ^{35}S-methionine into TCA-insoluble material was followed using the method of Baker et al.(1980). Briefly,

duplicate 0.5ml cultures were incubated for 1h in maintenance medium containing either 10uCi/ml ^{3}H-uridine or 20uCi/ml ^{35}S-methionine. 5ml of cold 10% TCA was added and the precipitate collected by filtration through GF-C glass fibre filters. Filters were washed twice with cold 10% TCA and once with cold ethanol then dried and counted in PPO-toluene scintillant.

Labelling of cells for analysis on polyacrylamide gels was carried out as described in Shuttleworth et al.,(1982). Cells were labelled at a density of 10^{7}cells/ml in GMEM lacking methionine to which 600uCi/ml ^{35}S-methionine had been added. Labelled proteins were prepared for electrophoresis as described in Shuttleworth et al.,(1982).

SDS-polyacrylamide gel electrophoresis.

One-dimensional SDS-polyacrylamide gel electrophoresis of ^{35}S-methionine labelled proteins was performed as described in Shuttleworth et al.(1982). 10-20%exponential gradient polyacrylamide gels were run with a 2cm stacking gel using the discontinuous buffer system of Laemmli (1970). After electrophoresis gels were fixed in several changes of 45%methanol, 10% acetic acid andthen dried and autoradiographed.

Molecular weight marker proteins were run in adjacent tracks and visualised by staining with Coomasie brilliant blue. The markers and their molecular weights were; cytochrome C, 12,000; carbonic anhydrase,30,000; phosphorylase b, 95,000; fumarase, 49,000;transferrin, 77,000.

Two-dimensional-polyacrylamide gel electrophoresis.

IEF and NEPHGE first-dimension gels were were prepared and run according to the methods of O'Farrell(1975) and O'Farrell et al.(1977) respectively. IEF gels were electrophoresed for 7200 Vh and were

found to have a pI gradient of 4.5-6.3. NEPHGE gels were electrophoresed for 1600 Vh . The first-dimension gels were equlibrated for 1h then frozen at -70°. Exponential gradient SDS-polyacrylamide gels were used for electrophoresis in the second dimension. These were run as described above except that 5cm stacker gel was used.

Second-dimension gels were fixed, dried and autoradiographed as described above.

SECTION 3 RESULTS

Section 3.1 The control of IFNmRNA stability in Namalwa cells

a) Introduction

Current thinking relating to the control of IFN production maintains that the rapid shut-off of IFN synthesis in poly(rI).poly(rC) induced fibroblasts involves the selective inactivation of IFNmRNA by a specific repressor mechanism which is initiated at, or shortly after, the induction of IFN synthesis (see section 1.2). The evidence supporting this repressor hypothesis is indirect and based entirely on the phenomenon of superinduction in poly(rI).poly(rC) induced fibroblasts. The time course of IFN production in Namalwa cells in many ways resembles that described for fibroblasts (see section 1.2). In particular the rapid shut-off of IFN synthesis in Namalwa cells shortly after induction as in fibroblasts is accompanied by a rapid decline in the amounts of functional IFNmRNA present in the cells. Although IFNmRNA has a very short half-life in Namalwa cells, it is translated and therefore stable for several days after microinjection into oocytes (Morser et al., 1979). This suggests that IFNmRNA stability is controlled in Namalwa cells, however since virus-induced IFN production cannot be superinduced in these cells, no evidence has been obtained to support the involvement of a post-transcriptional repressor in the shut-off of IFN production.

The experiments described in this section investigate the control of IFNmRNA stability and its role in the regulation of IFN synthesis in Namalwa cells.

b) The effect of reduced incubation temperature on IFN production

During experiments on the secretion of IFN by Namalwa cells

(Morser and Colman, 1980) it was found that by reducing the temperature of incubation from 37° to 28° after induction, the overall yield of IFN at 24h after induction was paradoxically increased. This provided a potentially useful approach to studying the control of IFN synthesis, and the discovery prompted a series of experiments in which I collaborated with Dr Morser, with the assistance of Jane Oliver, the results of which have now been published (Morser and Shuttleworth, 1981).

In order to determine the optimum temperature causing increased yield of IFN at 24h, cells were induced and incubated at 37° before shifting to various temperatures at 7h after induction. Fig. 6 shows that the largest increase was caused when cells were shifted to 28°. Fig. 7 shows that the maximum effect was achieved when the temperature was reduced to 28° at 7h after induction, however increased yields resulted from a shift to 28° at any time during the first 10h of IFN synthesis, with the exception of 1h after induction. The small increase caused by incubating cells at 28° from the time of induction was reproducible, and in fact yields were still increased when the temperature was reduced up to 3h before induction. The increase in IFN yield at 24h caused by reducing incubation temperature to 28° at 7h after induction was on average 5-fold (mean of 16 experiments) and was observed in all but one experiment.

In these and subsequent experiments the cells were routinely treated with butyrate before induction. Table 3 shows that the increase in IFN yield caused by reducing incubation temperature was not affected by butyrate treatment, and likewise the increase in IFN yield caused by butyrate was not affected by the reduced temperature. Therefore butyrate treatment provided a useful way of increasing the amounts of IFN (and IFNmRNA) available for study.

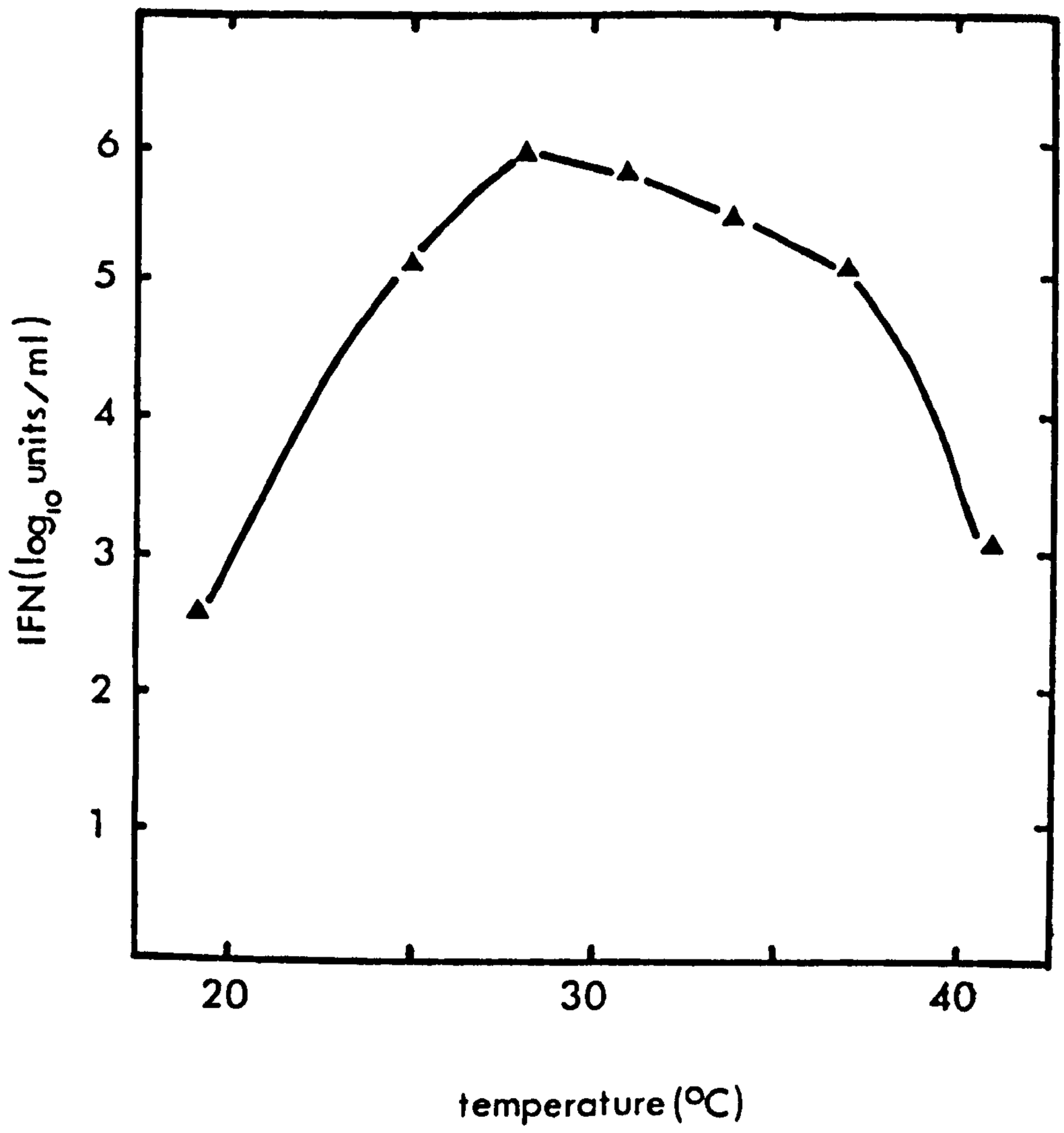


Fig.6. The effect of incubation temperature on IFN yield. Cells were induced at 37° and the temperature of incubation changed 7h later. The yield of IFN was assayed at 24h after induction. (Average of duplicate samples from a representative experiment.)

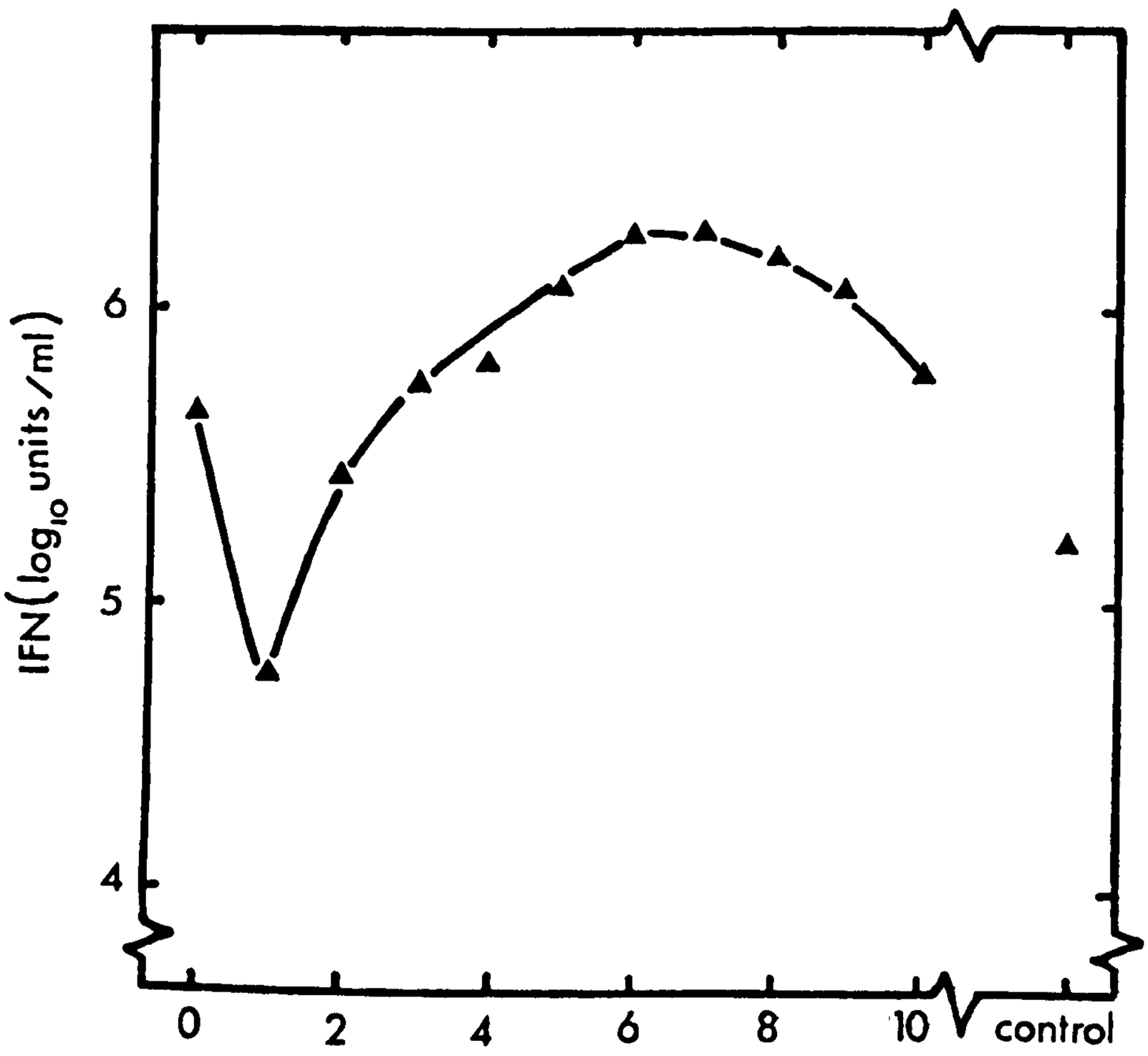


Fig.7. The effect on IFN yield of reducing the incubation temperature at different times. Cells were induced and incubated at 37° and their incubation temperature reduced to 28° at various times after induction. IFN yield was assayed at 24h after induction. Control value was obtained from cells incubated at 37° throughout. (Average of duplicate samples from a representative experiment.)

Table 3. The effect of reduced incubation temperature on IFN yields in butyrate-treated and untreated cells.

butyrate treatment	incubation temperature (°C)	IFN yield (log_{10}U/ml)		increase in IFN yield (x-fold) butyrate		28°	
		EBTr	IRA	EBTr	IRA	EBTr	IRA
+	37/28	5.55	4.75	11	14	25	22
-	37/28	4.50	3.61	-	-	22	16
+	37	4.15	3.40	10	10	-	-
-	37	3.15	2.40	-	-	-	-

Butyrate-treated and untreated cells were induced and incubated at 37°. At 7h after induction the incubation temperature of half of the cells was reduced to 28°. The IFN yield at 24h was determined by assay in bovine (EBTr) cells and by an immunoradiometric assay (IRA) using the monoclonal antibody NK2. IFN titres are expressed in international units together with the relative increase in IFN yield caused by butyrate and by the reduced incubation temperature. (Results from single samples assayed in duplicate.)

Several explanations could account for the effect of reduced incubation temperature on IFN yield. An effect on the intracellular degradation or secretion of IFN could be discounted since it was shown that the proportion of newly synthesized IFN being secreted (that is the ratio of intracellular to extracellular IFN) was not altered at 28° (Morser and Colman, 1980). Similarly the stability of secreted IFN was no different at 37° or 28°, therefore the increased yield was not due to decreased degradation of extracellular IFN (data not shown). Two remaining possibilities were investigated; firstly that the increased yield was not caused by increased synthesis of IFN but by a change in composition and specific activity of the IFNs produced. Secondly, that the regulation of IFN synthesis was altered at 28°, increasing the rate or production period of IFN synthesis.

i) Characterisation of the IFN produced at 28°

Several biological properties can be used to characterise IFN mixtures (see section 1.2), these include comparison of activities in heterologous and homologous cells and neutralization by monospecific antisera to IFN-α and IFN-β. No differences could be detected in the biological properties of IFN produced at 28° and 37° (data not shown), therefore there was no significant change in the composition of IFN produced at the lower temperature.

The Namalwa cells used in these experiments do not produce any detectable IFN-β activity (see section 2), consequently the increase in IFN yield at 28° must be caused by increased IFN-α synthesis. In order to confirm this, the amounts of IFN-α protein produced at 28° and 37° were compared by measuring the IFN in an immunoradiometric assay using the monoclonal antibody NK2 which is specific for a number of IFN-α species (which represent approximately

70% of the IFN-α activity produced by the cells, see section 2). Table 3 shows the effect of reduced temperature on IFN yield in untreated and butyrate-treated cells. Using a bioassay in EBTr cells, the increase in IFN activity was 22-fold for untreated and 25-fold for butyrate-treated cells, while the immunoradiometric assay detected a 16-fold and 22-fold increase in IFN respectively. Therefore at 28° the increased yield of IFN was explained by increased synthesis of IFN and was not due to changes in the specific activity of IFN. (The difference in IFN titre measured by bioassay and immunoradiometric assay is considered in section 2 and does not alter the interpretation of these data).

(ii) The kinetics of IFN production at 28°

Incubation of cells at 28° would be expected to reduce both the rate of general protein synthesis and IFN synthesis in which case IFN synthesis would have to continue for longer in order to result in equal or increased yields. To investigate this possibility, the rate of IFN synthesis and accumulation in the medium was measured using cells shifted to 28° at 7h after induction. It was already known that the proportion of newly synthesised IFN being secreted was not affected by the reduced temperature (Morser and Colman, 1980), so the rate of IFN synthesis was estimated by measuring the IFN secreted by cells which had been pelleted and resuspended in fresh medium. Although this procedure could have disturbed the normal rate of synthesis and secretion, it still allowed comparison of cells at 28° and 37°. Fig. 8a shows the amounts of IFN secreted over a 1h period at various times after induction. The rate of synthesis in cells at 37° continued to increase up to 13-14h after induction, then declined rapidly. By comparison, the rate of IFN synthesis in cells shifted to 28°,

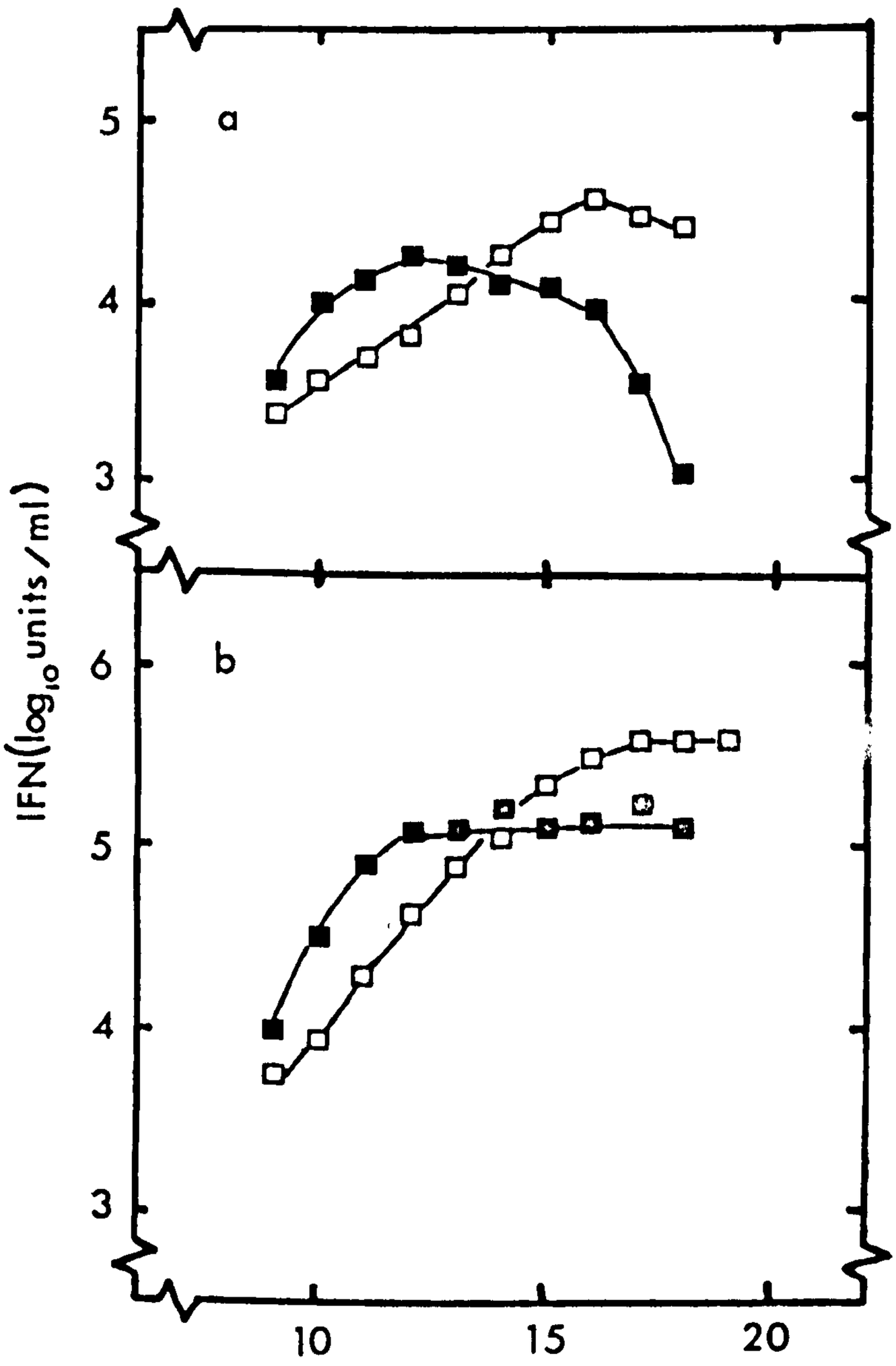


Fig.8. IFN production in cells incubated at 37° and 28°. Cells were induced and incubated at 37° for 7h. a) The rate of IFN synthesis* and b) the cumulative yield of IFN were measured in cells incubated at 37° (■) or at 28° (□) from 7h after induction. (Single samples from a representative experiment.)

* The amount of IFN secreted during a 1h period as described in text.

although initially slower than at 37°, continued to increase until 18h after induction exceeding the rate at 37°. Fig. 8b shows that the accumulation of IFN in the medium of cells at 37° had apparently ceased by 13-14h whereas with cells at 28° the accumulation was slower but continued for longer, resulting in a higher yield of IFN. The shut-off of IFN synthesis was therefore delayed in cells incubated at 28°.

The effect of the reduced incubation temperature on macromolecular synthesis was investigated by measuring the incorporation of ^{35}S-methionine and ^{3}H-uridine into TCA-insoluble material in cells shifted to 28° at 7h after induction. Fig. 9 shows that at 28° incorporation of methionine declined to 40% and uridine to 30% of the 37° control. Therefore the initial reduction in the rate of IFN synthesis at 28° (Fig. 8a) was consistent with a reduction in the rate of general macromolecular synthesis. However, the elevated rate of IFN synthesis at 28° at later time points (Fig. 8a) was clearly paradoxical in the face of results showing a depressed rate of general protein synthesis.

c) <u>The control of amounts of IFNmRNA in cells at 28° and 37°</u>

One explanation for the delay in shut-off and the increase in rate of IFN synthesis in cells incubated at 28° was that the control of amounts of IFNmRNA was affected in these cells. To investigate this possibility, the relative amounts of IFNmRNA present in cells incubated at 28° and 37° were compared at various times after induction. Total RNA was extracted from the cells, microinjected into Xenopus oocytes and the translation of IFN activity was measured. Fig. 10b shows the normal decay of IFNmRNA activity from 10h after induction in cells incubated at 37° (see also section 3.2). In cells shifted to 28°, amounts of IFNmRNA

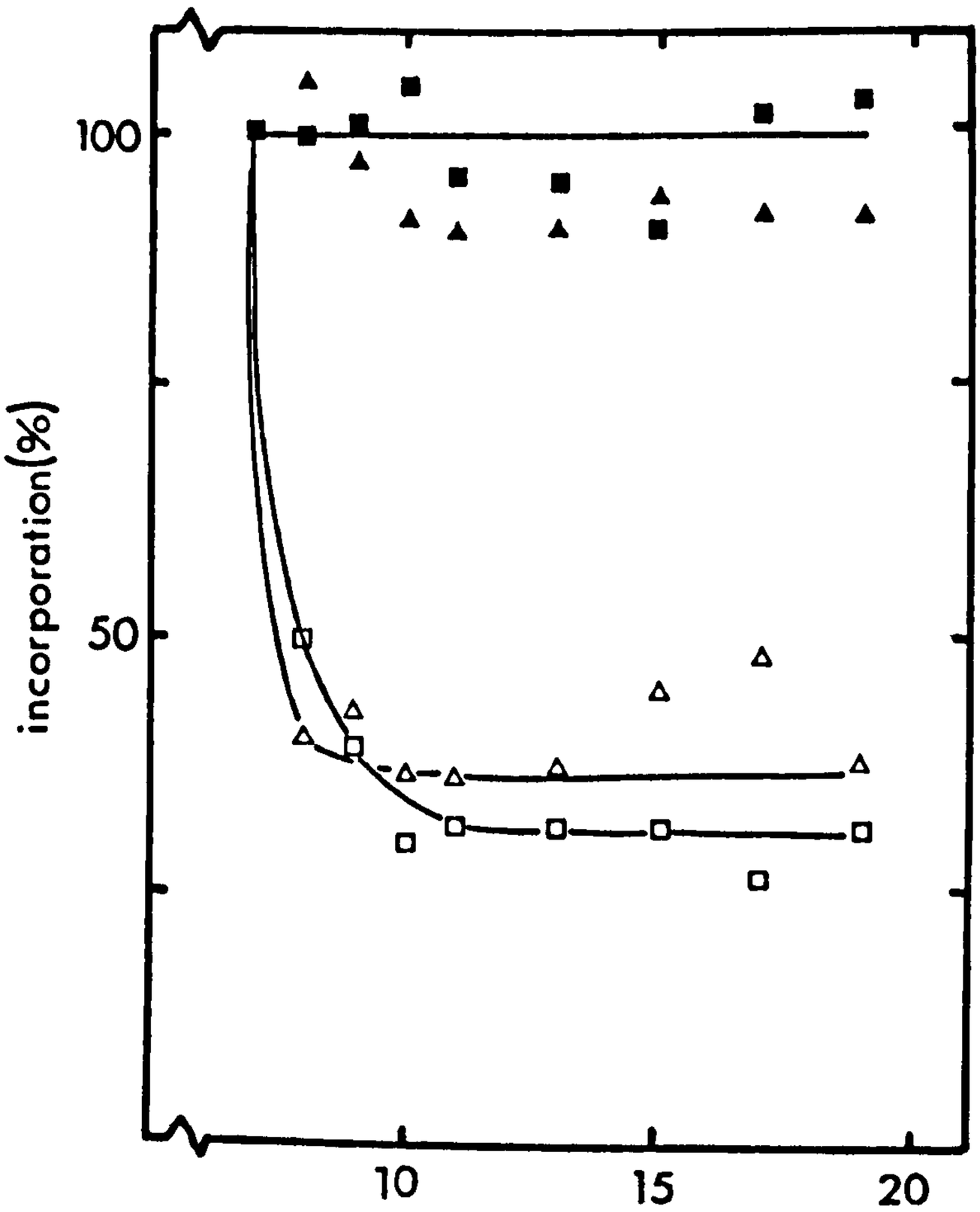


Fig.9. Rates of incorporation of ^{35}S-methionine and ^{3}H-uridine in cells incubated at 37° and 28°. Cells were induced and incubated at 37° for 7h. The incorporation of ^{35}S-methionine and ^{3}H-uridine into TCA-insoluble material (during a 1h labelling period) was measured in cells incubated at 37° or at 28° from 7h after induction. ▲, methionine incorporation at 37°; Δ, methionine incorporation at 28°; ■, uridine incorporation at 37°; □, uridine incorporation at 28°. (Single samples from a representative experiment.)

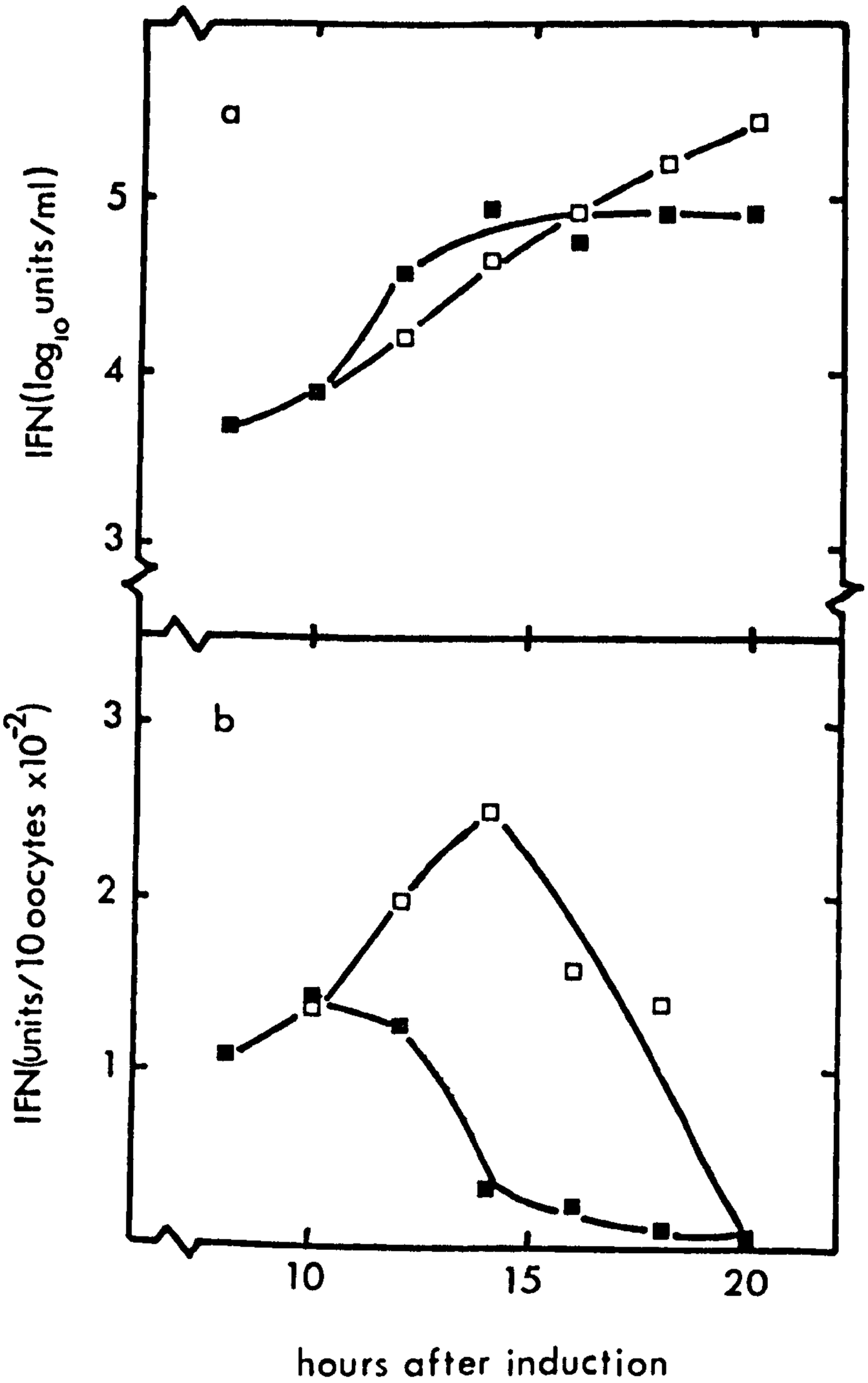


Fig.10. The cumulative yield of IFN and relative amounts of IFNmRNA in cells incubated at 37° and 28°. Cells were induced and incubated at 37° for 7h. a) The cumulative yield of IFN was measured in cells incubated at 37° (■) or at 28° (□) from 7h after induction. b) RNA was extracted from these cells and the relative amount of IFNmRNA determined by microinjection into Xenopus oocytes. The IFN translated from RNA from cells incubated at 37° (■) or at 28° (□) was then assayed. (Single samples from a representative experiment)

were initially comparable to those in cells at 37°. However at later times more IFNmRNA was present in cells at the lower temperature, and the maximum amount of IFNmRNA in cells at 28° exceeded that found in cells at 37°. Fig. 10a shows the accumulation of IFN in the medium of cells at both temperatures (from the same experiment) confirming that IFN synthesis continued for longer and resulted in a higher yield at 28°.

The RNA samples used in Fig. 10b were also assayed for the presence of IFN-∝ mRNA by dot-blot hybridization with an IFN-∝2 cDNA probe (see section 3.3, Fig. 22). The results of the hybridization analysis confirmed the conclusions made from translation of IFNmRNA in oocytes, showing that the relative amounts of IFNmRNA in cells incubated at 28° exceeded those in cells at 37° and that the decline of IFNmRNA was delayed in cells at 28°. In addition, as the loss of hybridizable IFNmRNA accompanied the loss of functional IFNmRNA (Figs. 10 and 22) the inactivation of IFNmRNA was caused by its degradation.

Arguably, the IFNmRNA detected by oocyte translation or by hybridization need not necessarily be involved in translation of IFN in the cell. It has been suggested that IFNmRNA can be detected in cells after IFN synthesis has apparently ceased (Berger et al., 1980). The data in Figs. 8 and 10 disagree with this view. IFN synthesis, measured by its rate of secretion, continued as long as IFNmRNA was present in the cells at both 28° and 37°. However, by measuring cummulative yield, as Berger et al. did, the small amount of IFN accumulating from this late IFN synthesis would not easily be detected on a logarithmic scale. In order to establish whether the IFNmRNA detected in cells late after induction was still associated with polysomes and capable of being translated, polysomes were prepared from cells,

their RNA extracted and microinjected into oocytes. Table 4 shows that not only was IFNmRNA still associated with polysomes at 14 and 16 hours after induction, but also that more IFNmRNA was associated with polysomes from cells incubated at 28° than cells at 37°. These results were therefore consistent with the finding that IFN synthesis continued for longer at 28°.

It remained to establish the reason why amounts of IFNmRNA, and hence rates of IFN synthesis at 28°, exceeded those at 37°. The most obvious explanation, that this was the result of continued transcription of IFNmRNA at 28°, was eliminated by inhibiting RNA synthesis by the addition of actinomycin D before reducing the incubation temperature. Table 5 shows clearly that when actinomycin D was added and the incubation temperature reduced to 28° at 3.5h after induction, an increase in IFN yield still occurred. In fact the increase in IFN yield at 28° was greater in the presence of inhibitor. The reduced incubation temperature must therefore be affecting the stability of IFNmRNA synthesised before the temperature shift. This suggested that IFNmRNA which would normally be degraded in cells at 37° persists in cells at 28°. As a consequence this increased accumulation of IFNmRNA and delay in its degradation could explain the prolonged period of synthesis and increased yield of IFN in cells incubated at 28°. This does not eliminate the possible involvement of continued IFNmRNA synthesis in causing increased yield at 28°, but only showed that it was not necessary.

d) <u>The mechanism of IFNmRNA inactivation</u>

The fact that IFNmRNA can be translated for several days after microinjection into oocytes suggests that its rapid degradation shortly after the induction of IFN production in Namalwa cells is caused by an active process which is absent in Xenopus oocytes.

Table 4. The amount of IFN$_m$RNA present on polysomes during the shut-off of IFN synthesis.

hours after induction	incubation temperature (^{o}C)	IFN yield from polysomal RNA (units/ml)
14	37	40
14	37/28	125
16	37	16
16	37/28	25

RNA was extracted from polysomes prepared from cells incubated at 37^{o} throughout or at 28^{o} from 7h after induction. The RNA was microinjected into oocytes and the IFN produced was assayed. (Single samples from a representative experiment.)

Table 5. The effect of actinomycin D on the enhancement of IFN yield caused by reduced incubation temperature.

incubation temperature (^{o}C)	IFN yield (log_{10}units/ml)	
	no inhibitor	+ actinomycin D
37	5.0	3.8
37/28	5.4	4.6

cells were induced and incubated at 37°. At 3.5h after induction actinomycin D (1ug/ml) was added to half of the cells. The IFN yield at 24h was compared in cells incubated at 37° throughout and cells reduced to 28° at 3.5h after induction, in the presence and absence of actinomycin D.(Average of duplicate samples from a representative experiment)

In poly (rl).poly(rC) fibroblasts this process has been attributed to a post-transcriptional repressor molecule which, because of the requirement for both transcription and translation, has been suggested to be a protein. The following preliminary experiments were designed to test whether a similar repressor mechanism exists in Namalwa cells which could be reconstructed in a heterologous system, namely Xenopus oocytes.

The induction and accumulation of the hypothetical repressor has been used to explain both the inactivation of IFNmRNA and the hyporesponsive or refractory state (see section 1.2). Because the repressor is assumed to be induced at the same time as IFN, two questions were asked. Firstly, could the mRNA coding for such a protein be translated and shown to affect the stability and hence the translation of IFNmRNA in oocytes, and if so, do the amounts of this mRNA alter during the induction and shut-off of IFN synthesis?

In the experimental approach used, total RNA lacking any detectable IFNmRNA activity was extracted from either non-induced cells or cells in the refractory state 18h after induction and injected together with RNA containing IFNmRNA extracted at 7h after induction. Table 6 shows that the amount of IFN translated by the oocytes was not affected by either of the RNA samples from cells not producing IFN. In another experiment, similar RNA samples extracted from cells before and 18h after induction, were injected into oocytes 24h before injecting RNA containing IFNmRNA. Table 7 shows that again no effect on translation of IFN could be detected.

These negative results can be interpreted in several ways. Either the repressor does not exist, since neither RNA or protein translated from this RNA had the predicted effect on IFNmRNA translation, or it was possible that the oocyte did not provide the necessary environment for the mechanism to operate. Alternatively the mRNA or protein could be labile and rapidly lost from the oocytes.

Table 6. The effect of coinjection of RNA from non-induced and refactory cells on the translation of IFNmRNA by Xenopus oocytes.

RNA sample	IFN yield (log_{10}units/10oocytes)
non-induced	0
7h	0.40
18h	0
7h + non-induced	0.35
7h + 18h	0.40

Total RNA was extracted from cells before induction, 7h after induction and 18h after induction. These samples were then microinjected into Xenopus oocytes either individually or in the combinations indicated, and the yield of IFN translated during the following 24h was assayed.

(Results from one experiment, singles samples)

Table 7. The effect of preinjection of RNA from non-induced and refactory cells on the translation of IFNmRNA by Xenopus oocytes.

RNA sample injected at 0h	RNA sample injected at 24h	IFN yield at 48h (log_{10}units/10oocytes)
water	7h	2.65
non-induced	7h	2.65
18h	7h	2.65

Oocytes were microinjected with either water, total RNA extracted from non-induced cells or total RNA extracted from cells at 18h after induction. After incubating for 24h the oocytes were then microinjected with total RNA from cells at 7h after induction and the yield of IFN translated during a further 24h incubation was assayed. (Results from one experiment, single samples)

e) Discussion

The purpose of these experiments was to consider the control of IFNmRNA stability and its role in regulating IFN synthesis in Namalwa cells. The results presented clearly confirm that inactivation and degradation of IFNmRNA occurs during the shut-off of IFN synthesis. When the incubation temperature is reduced, IFN synthesis continues for longer and the degradation of IFNmRNA is delayed. However, interpretation of the effects of reduced incubation temperature on IFN production is difficult without data comparing the rates of IFNmRNA synthesis and degradation at 28° and 37°. The best way to obtain such measurements is by pulse-labelling nascent RNA and hybridization with specific IFNcDNA probes (see section 1.1). This approach was explored, however since IFNmRNA represents such a small proportion of total RNA (see section 3.3), labelling of intact cells with RNA precursors failed to incorporate sufficient radioactivity for these measurements to be made. Alternative strategies are discussed in section 4.

Bearing in mind that reduced incubation temperature most probably affects both the rates of macromolecular synthesis and degradation, the effect of incubating cells at 28° on amounts of IFNmRNA, IFN yield and period of IFN synthesis can be interpreted in several ways. The reduced incubation temperature could have a non-specific effect, reducing the rate at which all mRNA's are degraded, thus explaining the persistance of IFNmRNA. Balanced by this however, the reduced rate of RNA and protein synthesis at 28° would be unlikely to result in the observed effects of reduced temperature on amounts of IFNmRNA, rates of IFN synthesis and IFN yield, all of which eventually exceed the values attained by cells at 37°. Alternatively, the reduced temperature could differentially affect a mechanism controlling IFNmRNA stability,

for example, by reducing the rate of synthesis of a hypothetical repressor molecule. Several observations favour the latter interpretation. i) The timing of the temperature shift was restricted to the first 10h of IFN production for increased IFN yields to occur. If the effect of reduced temperature was general, then an increase should have occurred when cells were shifted to 28° at any time during the period of IFN production. ii) The amounts of IFNmRNA and rates of IFN synthesis in cells at 28° continued to rise above those at 37°, indictating that either IFNmRNA synthesis was continuing for longer, or that its degradation was inhibited at 28°. iii) Continued transcription and IFNmRNA synthesis was not however necessary to cause increased IFN yields at 28°.

Using the post-transcriptional repressor model as a working hypothesis, these results can be explained as follows. Perhaps the simplest scenario suggested by the effects of metabolic inhibitors on poly(rI).poly(rC) induced fibroblasts is one in which IFN synthesis precedes repressor synthesis, although both are induced at (or around) the same time. Once IFNmRNA synthesis slows down, the rising repressor levels begin to increase the rate of degradation of IFNmRNA, causing a decline in its amounts (see section 1.2). When the accumulation of repressor is prevented, by inhibiting protein and RNA synthesis, IFNmRNA persists resulting in increased IFN yields. By analogy, I would propose that by reducing the incubation temperature of Namalwa cells, repressor synthesis is reduced. As a consequence any IFNmRNA produced before or after temperature shift would be stabilised. Therefore increased IFNmRNA synthesis would not be required to explain the increased amounts of IFNmRNA present in cells at 28°, although continued synthesis would obviously contribute to this. By 10h after induction the rate of

IFNmRNA synthesis has probably declined and sufficient repressor could be accumulated, preventing any such effect occurring. This explanation is strengthened by the fact that reduced incubation temperature has a similar effect on IFN production in poly(rI).poly(rC) induced fibroblasts (Havell and Vilcek, 1973) however it remains speculative.

In conclusion the shut-off of IFN synthesis in Namalwa cells is accompanied and probably caused by the degradation of IFNmRNA. However, the possibility that degradation of IFNmRNA is a consequence of the shut-off of IFN synthesis cannot be eliminated. The data obtained by comparing amounts of IFNmRNA, rates of IFN synthesis and IFN yield in Namalwa cells incubated at 37° and 28° are consistent with the hypothesis that IFNmRNA is degraded by a mechanism (possibly a repressor) which is selectively inhibited by reducing the incubation temperature. These results and the phenomenon of superinduction are open to other interpretations which are discussed in section 4.

Section 3.2 Control of IFNmRNA levels and IFN yields: the effects of butyrate and BrdUrd

a) Introduction

The previous section has considered how control of IFNmRNA stability may be involved in regulating the shut-off of IFN production in Namalwa cells. However, despite the fact that the period of IFN production is tightly controlled and does not vary significantly from one occas ion to another, IFN yield can vary by over two orders of magnitude, even when cell culture, induction and assay conditions are strictly controlled and standardised. One obvious possibility is that rates of transcription or processing of IFNmRNA are controlled. However, the relationship between the amount of IFNmRNA present in the cells and IFN yield has received little attention. In order to investigate its cause and possible significance, the natural variation in IFN yield could be exploited by looking for similar variation in relative amounts of IFNmRNA. An alternative approach would be to investigate the increase in IFN yields caused by treating Namalwa cells with butyrate or BrdUrd before induction. These agents do not alter the time course of IFN production (Baker et al., 1979; 1980), and butyrate has already been shown to increase IFN yield by increasing the rate of IFNmRNA synthesis (Morser et al., 1980).

This section describes the effect of butyrate and BrdUrd treatments on the amounts of IFNmRNA synthesised by the cells and considers how these treatments cause increased yields of IFN. These results have now been published (Shuttleworth et al., 1982).

b) IFNmRNA levels in butyrate- and BrdUrd-treated cells

Treatment of Namalwa cells with 0.8 mM butyrate or 25 μg/ml BrdUrd for 48 h before induction reproducibly increased the yield of

IFN produced 24 h after induction. The effect of these treatments on the relative amounts of IFNmRNA synthesised by the cells was investigated by extracting total RNA from treated and untreated cells at 8 h after induction. The RNA was microinjected into Xenopus oocytes and the IFN activity translated was used as a measurement of the relative amount of IFNmRNA present in the cells. Fig. 11a shows the yields of IFN produced by these cells at 8 h after induction (results from 16 experiments) from which it can be seen that both treatments caused an increase in IFN yield. Although the yield varied (for untreated cells, 2.35-5.10; for butyrate-treated cells, 3.95-6.10; for BrdUrd-treated cells, 2.95-4.95 Log_{10} units/10^6cells) treatment caused an increase in every experiment. The size of this increase, calculated from the increase in each experiment, varied from 8-300 fold and averaged 77-fold for butyrate-treated cells. For BrdUrd-treated cells the increase varied from 3-35 fold and averaged 11-fold. Fig. 11b shows that both treatments also caused an increase in the relative amounts of IFNmRNA in the cells (from the same 16 experiments), The average increase, calculated from the increase in each experiment, was 26-fold for butyrate-treated and 3-fold for BrdUrd-treated cells. The increase in IFN yield was therefore 3-4 fold greater than the increase in amounts of IFNmRNA. In every experiment, butyrate-treated cells produced more IFN and IFNmRNA than did BrdUrd-treated cells, both producing more than untreated cells. Therefore the increased production of IFN was always accompanied by, but not due solely to, an increase in amounts of IFNmRNA.

Considering the range of values obtained for IFN yield and IFNmRNA from treated and untreated cells, it was necessary to evaluate the significance of the increases caused by butyrate and BrdUrd treatment. There are several sources of error which could contribute to this variation, apart from differences in cell culture

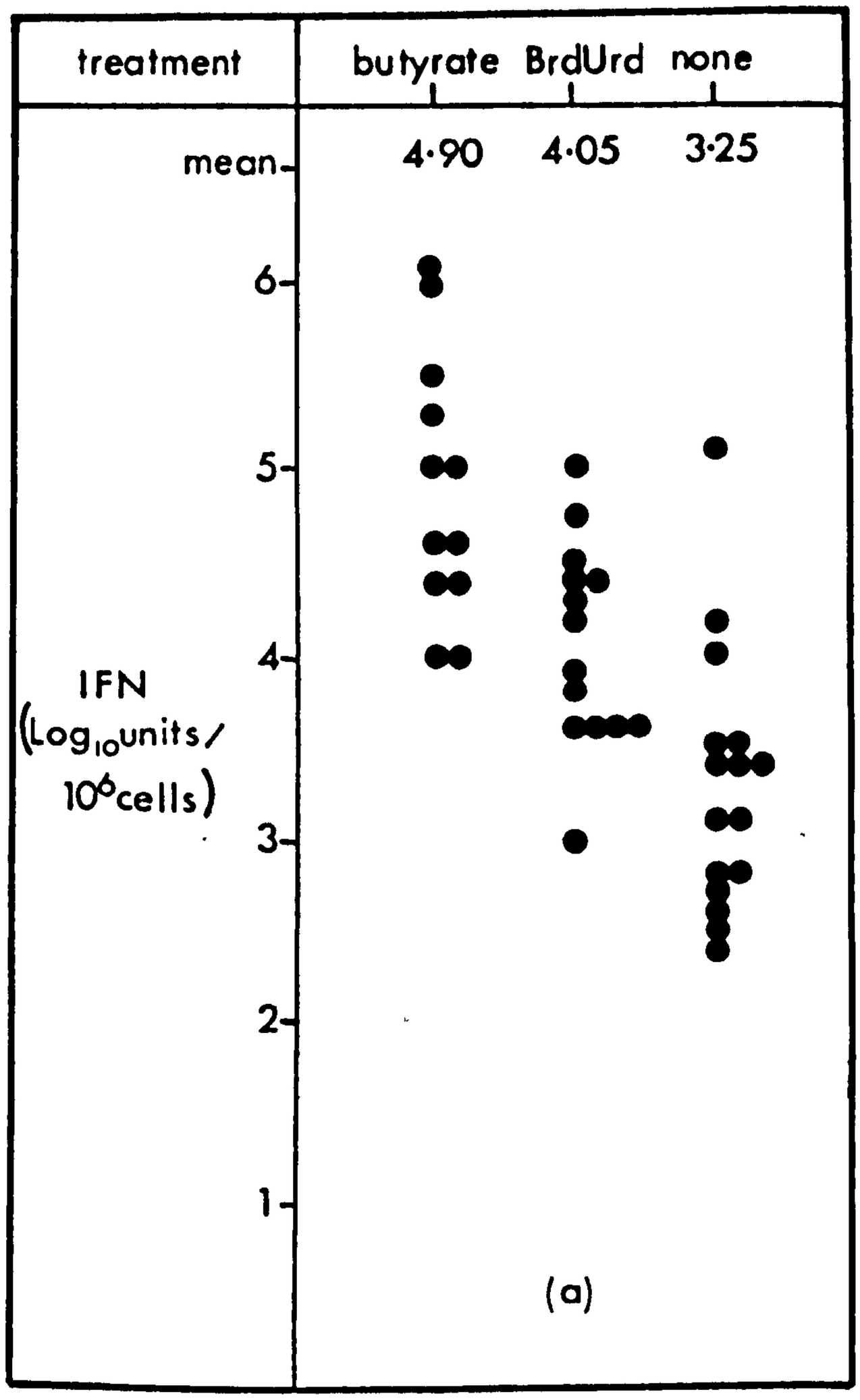


Fig.11a. The effect of butyrate and BrdUrd on IFN yield. Cells were incubated in 0.8 mM butyrate, 25 ug/ml BrdUrd or maintenance medium for 48h, then induced. At 8h after induction the cumulative IFN yield was measured. The range and mean of values obtained from 16 experiments are shown.

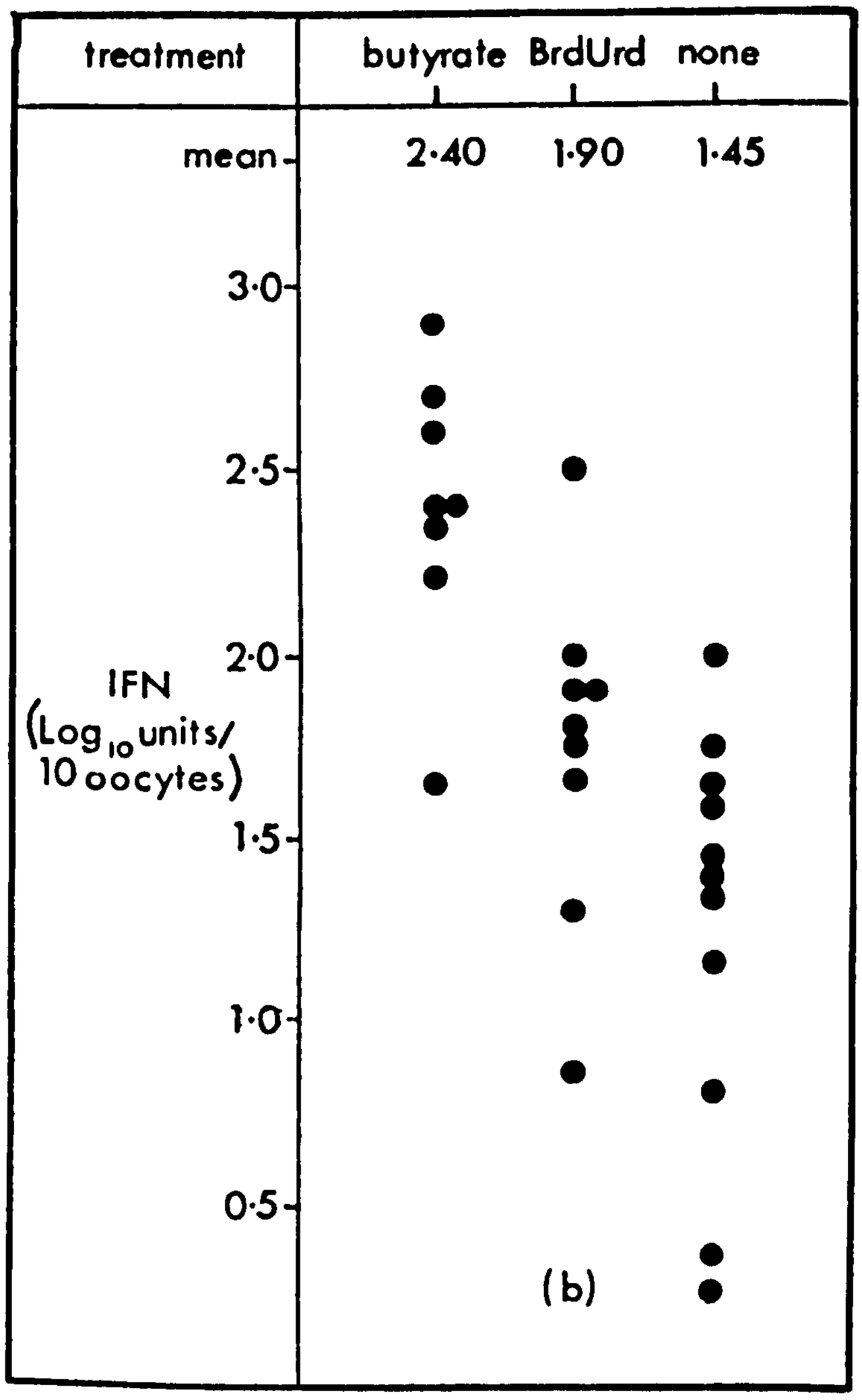


Fig 11b. The effect of butyrate and BrdUrd on IFNmRNA levels. RNA was extracted from the cells used in Fig 11a, and microinjected into oocytes. The IFN translated was used to determine the amounts of IFNmRNA present. The range and mean of values obtained from 11 experiments are shown.

conditions, media and handling (which were minimised by strict experimental protocols). For example, the IFN assay itself was subject to variation for the same sample assayed on different occas ions (interassay variation), differed by up to 2-fold (i.e. $\pm$ 0.3 Log_{10} Units). This variation was corrected for by including an internal standard with each assay. Intra-assay variation was found to be considerably lower. Similarly, it was necessary to include an internal standard IFNmRNA preparation in oocyte microinjection assays, since the efficiency of translation of RNA samples was found to vary by up to 4-fold (i.e. $\pm$ 0.6 Log_{10} Units) when using different batches of oocytes. In order to test the significance of the increases in IFN yield and amounts of IFNmRNA caused by treatment, a one-way analysis of variance test was applied to the data in Figs. 11a and 11b with the null hypothesis being that the distribution of values for treated cells could be accounted for by the variance of data from untreated cells, the probability of obtaining these results by chance was less than 0.1% (i.e. $P<0.001$). Therefore treatment was significantly affecting both IFN yield and amounts of IFNmRNA.

c) <u>Characterisation of IFN produced by treated cells</u>

Bearing in mind the points raised in sections 1.2 and 3.1 regarding the specific activity of IFN-α species, it was necessary to confirm that the increase in IFN yields caused by butyrate and BrdUrd was due to increased IFN synthesis and not a change in the composition of the IFN produced. To do this, the IFN produced by butyrate-treated, BrdUrd-treated and untreated cells was measured by bioassay in bovine EBTr cells, human cells and also by the immunoradiometric assay using the NK2 monoclonal antibody to IFN-α . Table 8 shows that a similar increase was measured in both IFN activity and antigenic

Table 8. Characterisation of the IFN produced by treated and untreated cells.

treatment	IFN (log_{10}units/10^6cells)		
	EBTr	HFF	IRA
butyrate	4.40 (38)	4.60 (19)	3.10 (22)
BrdUrd	3.60 (6)	3.75 (3)	2.60 (7)
none	2.80	3.30	1.75

Cells were treated for 48h with either 0.8mM butyrate or 25ug/ml BrdUrd or maintainance medium. At8h after induction the cumulative IFN yield was assayed using EBTr cells, HFF cells or by an. immmunoradiometric assay (IRA). Titres are all expressed in international units. Figures in parentheses indicate the relative enhancement in IFN yield from treated cells.

mass produced by treated cells (see also section 3.1, table 3). These Namalwa cells do not produce active IFN-β (see section 2 and 3.3) therefore these results indicated that butyrate and BrdUrd treatment caused a genuine increase in IFN-α synthesis. Similarly, when the IFN translated in oocytes from RNA extracted from butyrate-treated and untreated cells was measured by bioassay and by the immunoradiometric assay, the relative increase in IFN activity corresponded with a similar increase in antigenic mass (data not shown). Furthermore the relative increase in amounts of IFNmRNA in RNA from butyrate- and BrdUrd-treated cells was measured by hybridization with IFNcDNA probes and found to be similar to the increase measured by oocyte translation (see section 3.3). This eliminated the possibility that the IFNmRNA from treated cells was for some reason translated less efficiently in oocytes than IFNmRNA from untreated cells, an effect which would result in the underestimation of amounts of IFNmRNA in treated cells. Therefore the increase in IFNmRNA content of treated cells was not sufficient to account for the increase in IFN yield.

d) <u>Are the effects of treatment on IFNmRNA levels and IFN yields causally related</u>?

If the increase in IFN yields was caused, at least in part, by the effect of treatment on the rate of IFNmRNA synthesis, then changes in conditions of treatment should result in parallel effects on both the amounts of IFNmRNA and IFN yields. It had previously been shown that by altering the concentration of butyrate, the effect on the increase in IFN yield and amounts of IFNmRNA correlated exactly, the optimum concentration being around 1mM (Morser et al., 1980). Figs. 12a and 12b show that likewise, when the cells were treated for 48 h with various concentrations of BrdUrd, the increases in IFN yield and IFNmRNA at 8 h after induction were similarly

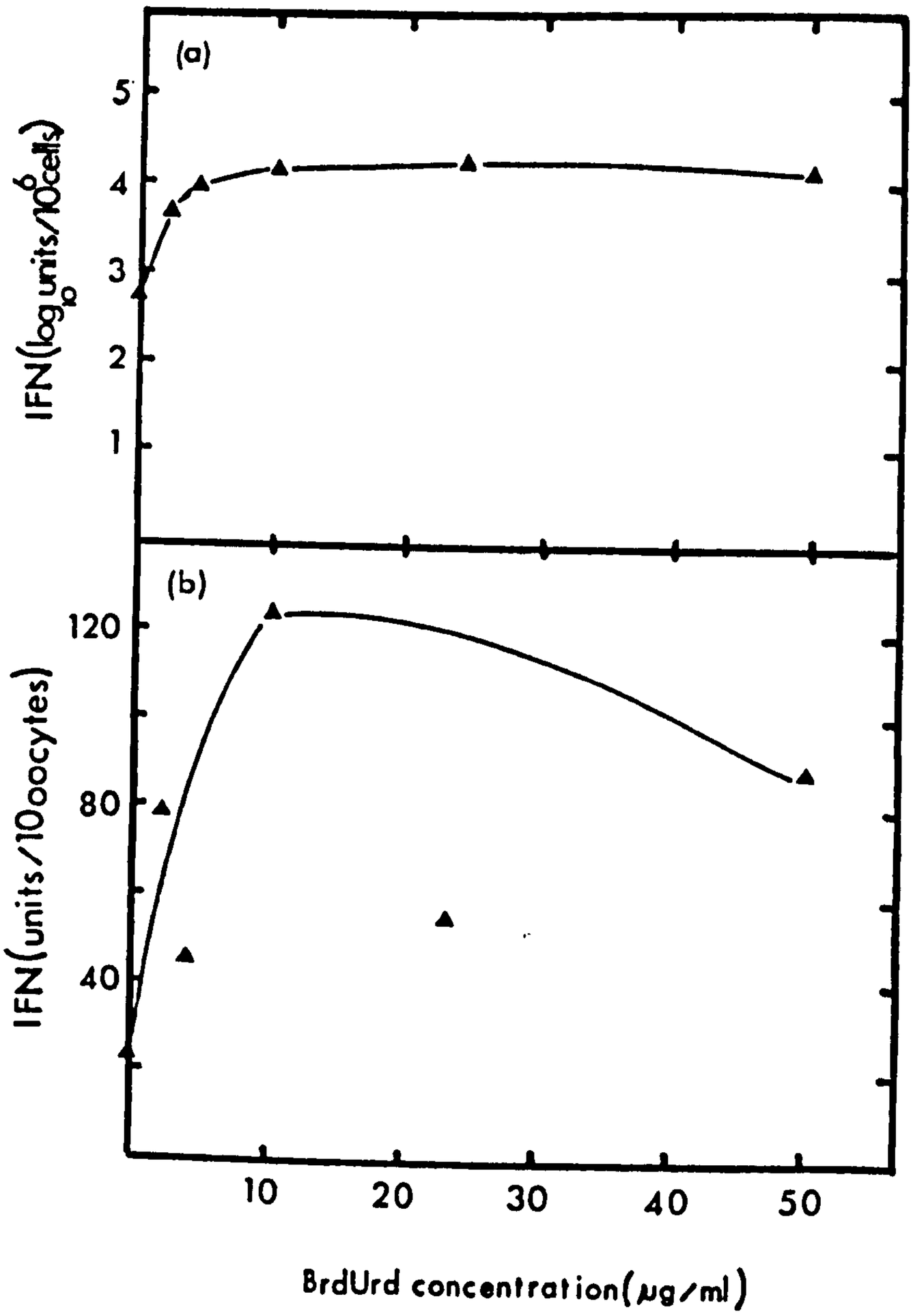


Fig.12. The effect of varying the concentration of BrdUrd on IFN yields and IFNmRNA levels. Cells were treated for 48h with various concentrations of BrdUrd, then induced. a) The cumulative IFN yield at 8h after induction was assayed. b) The amount of IFN translated by oocytes microinjected with total RNA extracted from the cells at 8h after induction.
(single samples from a representative experiment)

affected. Both IFN yield and amounts of IFNmRNA increased rapidly when the concentration was raised from 0-10ug/ml, then gradually declined as the concentration was increased further.

The same observations were made using cells which had been treated for different times before induction. Cells were suspended in maintenance medium and either 0.8mM butyrate or 25ug/ml BrdUrd added at various times from 4-48h before induction. IFN yield and amounts of IFNmRNA were measured at 8h after induction. Figs. 13a and 13b show that increases in both yield of IFN and amounts of IFNmRNA were detected after only 4h of treatment with either agent, and the effects increased, reaching a maximum after 48h of treatment. Again, on all occassions the increase in IFN yields exceeded the increase in amounts of IFNmRNA, therefore thetreatments cannot be affecting the rate of IFNmRNA synthesis alone.

Since it was possible that butyrate and BrdUrd affect IFN synthesis at different points, the following experiment was designed to test whether combined treatment had an additive effect on IFN yield. Simultaneous treatment would be unlikely to have any such effect because the inhibition of DNA synthesis by butyrate (Adolf and Swetley, 1979) would prevent the incorporation of BrdUrd which is required to increase IFN yield (Baker et al., 1979). Various regimens of sequential treatment were used and as Table 9 shows, no additive effects on IFN yield or amounts of IFNmRNA were detected. Therefore, the mechanisms by which these agents affect IFN synthesis are either mutually exclusive, mediated by common pathways or restricted by another rate limiting process. Surprisingly though, these conditions of treatment resulted in increases in IFN yields which were equivalent to the increases in amounts of IFNmRNA. It would appear that cells which had been held in maintenance medium for 48h before treatment were altered such that butyrate or BrdUrd

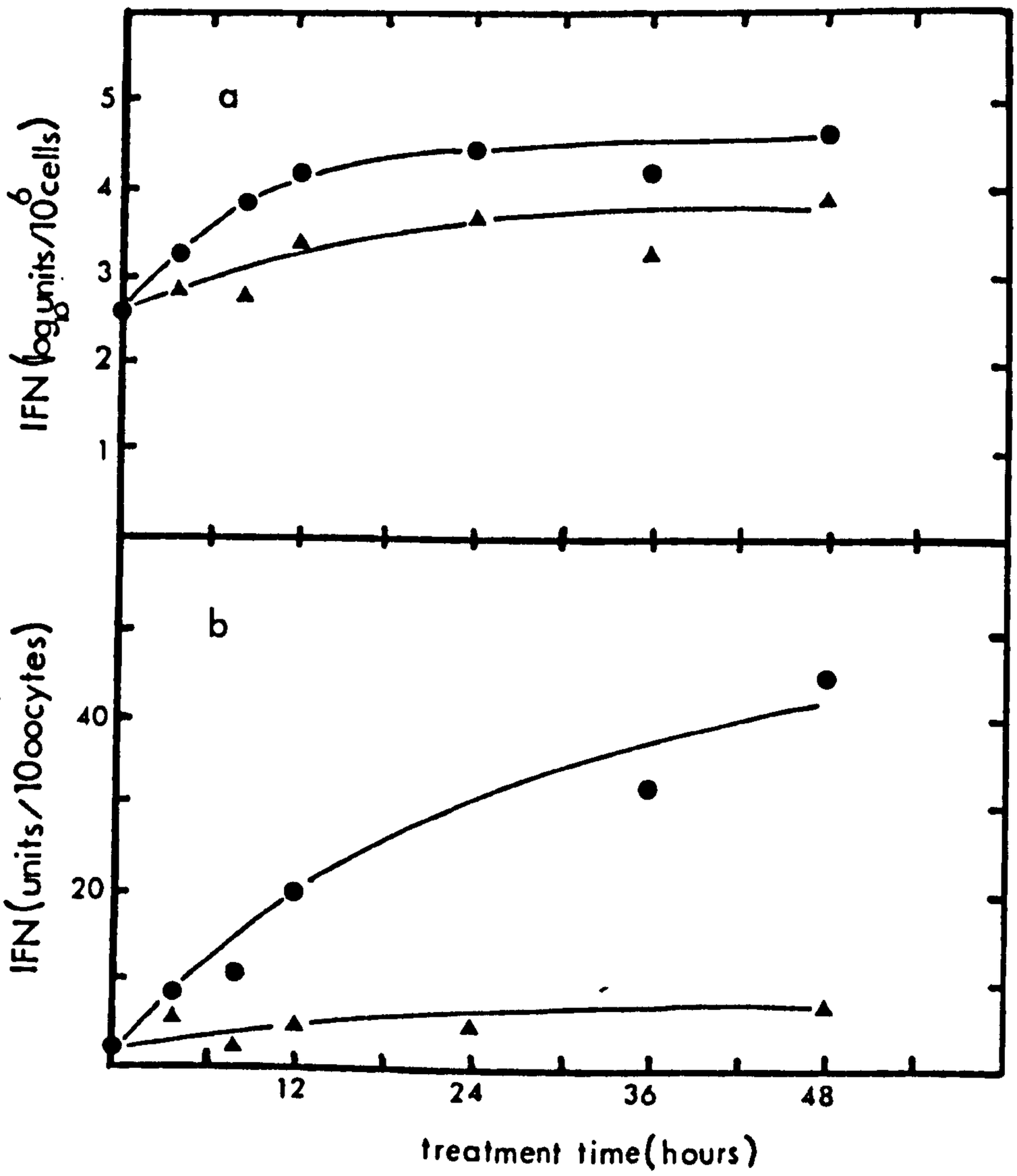


Fig. 13. The effect of varying times of treatment on IFN yield and amounts of IFNmRNA. The cells were treated for various times with either butyrate (●) or BrdUrd (▲) then induced.

(a) The cumulative IFN yield at 8h after induction.

(b) The amount ofIFN secreted by oocytes microinjected with total RNA extracted from cells at 8h after induction.

(single samples from a representative experiment)

Table 9. The effect of sequential treatment with butyrate and BrdUrd on IFN yield and IFNmRNA levels.

treatment		IFN	IFNmRNA
0-48h	48-96h	(log_{10}units/10^6cells)	(log_{10}units/10oocytes)
BrdUrd	butyrate	4.20 (12)	2.20 (11)
BrdUrd	none	3.70 (4)	1.60 (3)
butyrate	none	3.50 (3)	1.65 (3)
none	butyrate	4.40 (20)	2.40 (18)
none	BrdUrd	3.80 (5)	1.65 (3)
none	none	3.10	1.15

Cells were treated with 0.8 mM butyrate, 25ug/ml BrdUrd or maintenance medium for the times indicated, then induced. At 8h after induction the cumulative IFN yield was measured. Total RNA was extracted and microinjected into oocytes and the amount of IFN translated used to determine therelative amounts of IFNmRNA present in the oells. Figures in parentheses indicate the relative increases in treated cells.

(single samples from a representative experiment)

treatment no longer caused an increase in IFN yield which was disproportionate to the increase in amounts of IFNmRNA.

e) The time course of IFNmRNA and IFN synthesis

The time course of IFN production has been reported to be unaffected by butyrate or BrdUrd treatment (Baker et al., 1979; 1980), therefore it was unlikely that the difference between the increase in relative amounts of IFNmRNA and the increase in IFN yields in treated and untreated cells was caused by a more rapid decline in the amounts of IFNmRNA in treated cells so that by the time at which IFN yield was measured (8h after induction) IFNmRNA levels were low. However this possibility was tested by comparing the amounts of IFNmRNA and cumulative IFN yield at various times after induction in butyrate-treated, BrdUrd-treated and untreated cells. Fig. 14a confirms that the treatments did not alter the time course of IFN production, and the increase in IFN yield was seen at all times for both treatments. Total RNA was extracted from the cells at these times and translated in oocytes. Fig. 14b shows that both treatments caused an increase in the amounts of IFNmRNA at all times after induction. Amounts of IFNmRNA accumulated to a maximum at 9h after induction, then declined with a half-life of approximately 2.7h in both treated and untreated cells. (In fact the decline in IFNmRNA levels had an average half-life of 2.7h in 18 other experiments with treated and untreated cells.) Therefore treatment increased the rate of IFNmRNA synthesis and inactivation, but at no time did the increase in amounts of IFNmRNA completely account for the increase in IFN yield.

f) Intracellular IFN and its rate of secretion

Extracellular yields of IFN need not necessarily reflect the amount of IFN synthesised by the cells. If, for example, treatment

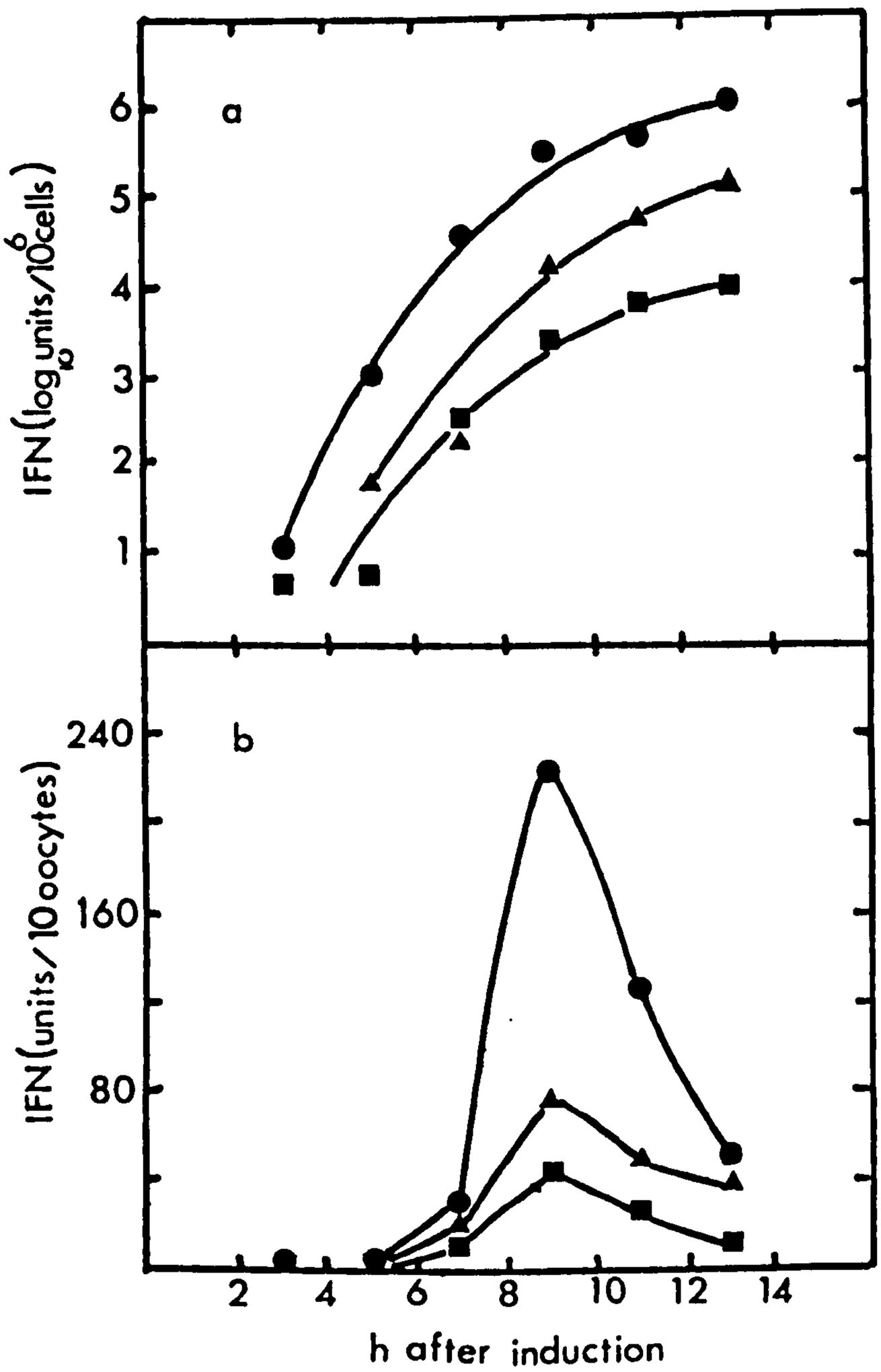


Fig.14. The time course of accumulation of IFN and IFNmRNA in treated and untreated cells. Cells were incubated with butyrate (●), BrdUrd (▲) or maintenance medium (■) then induced.

(a) The cumulative IFN yield was assayed at various times after induction.

(b) RNA was extracted from the cells at various times after induction and microinjected into oocytes. The IFN translated was assayed in order to determine the amounts of IFNmRNA.

(single samples from a representative experiment)

reduced the intracellular accumulation or degradation of IFN, or increased its rate of secretion, then this could cause a disproportionate increase in extracellular yield compared to the increase in amounts of IFNmRNA. For this reason the intracellular levels of IFN, its rate of secretion and extracellular accumulation were compared in treated and untreated cells.

Cells were treated for 48h with butyrate or BrdUrd, induced, then after 8h they were pelleted and the supernatant used to determine cumulative extracellular yield of IFN. The cells were washed and resuspended in fresh medium for 1h, and the amount of IFN secreted was measured. Intracellular IFN was estimated in washed cells at the same time. Table 10 shows the effect of butyrate and BrdUrd on each of these parameters, and clearly intracellular levels, rate of secretion and extracellular yield are equally increased. Therefore, the extracellular yield of IFN accurately reflected the relative amounts of IFN synthesised, and treatment was not altering the post-translational movements of IFN.

g) <u>The effect of treatment on translation of IFNmRNA</u>

Having confirmed that the increase in IFN synthesis in butyrate and BrdUrd treated cells was greater than the increase in IFNmRNA, the most likely explanation was that treatment caused an increase in the efficiency of IFNmRNA translation. The effect was not caused by an increase in the rate of general protein synthesis, since as Fig. 15 shows, the rate of incorporation of ^{35}S-methionine into TCA-insoluble material following induction was if anything lower in treated cells. In butyrate-treated cells ^{35}S-methionine incorporation was approximately 96% of that in untreated cells, therefore the 60% inhibition of protein synthesis caused by 48h treatment with butyrate (Baker et al., 1980) was rapidly reversed

Table 10. Intracellular IFN, its rate of secretion and extracellular accumulation in treated and untreated cells.

	IFN (log_{10}units/10^6cells)		
treatment	cumulative extracellular 8h	secreted 8-9h	intracellular 8h
butyrate	4.40 (38)	4.10 (16)	2.40 (18)
BrdUrd	3.60 (6)	3.60 (5)	1.85 (5)
none	2.80	2.90	1.15

Cells were treated for 48h with either 0.8mM butyrate or 25ug/ml BrdUrd or maintenance medium. At 8h after induction the cumulative IFN yield and intracellular levels of IFN were measured, together with the amount of IFN secreted from 8-9h after induction. Figures in parentheses indicate the relative enhancement of IFN in treated cells.

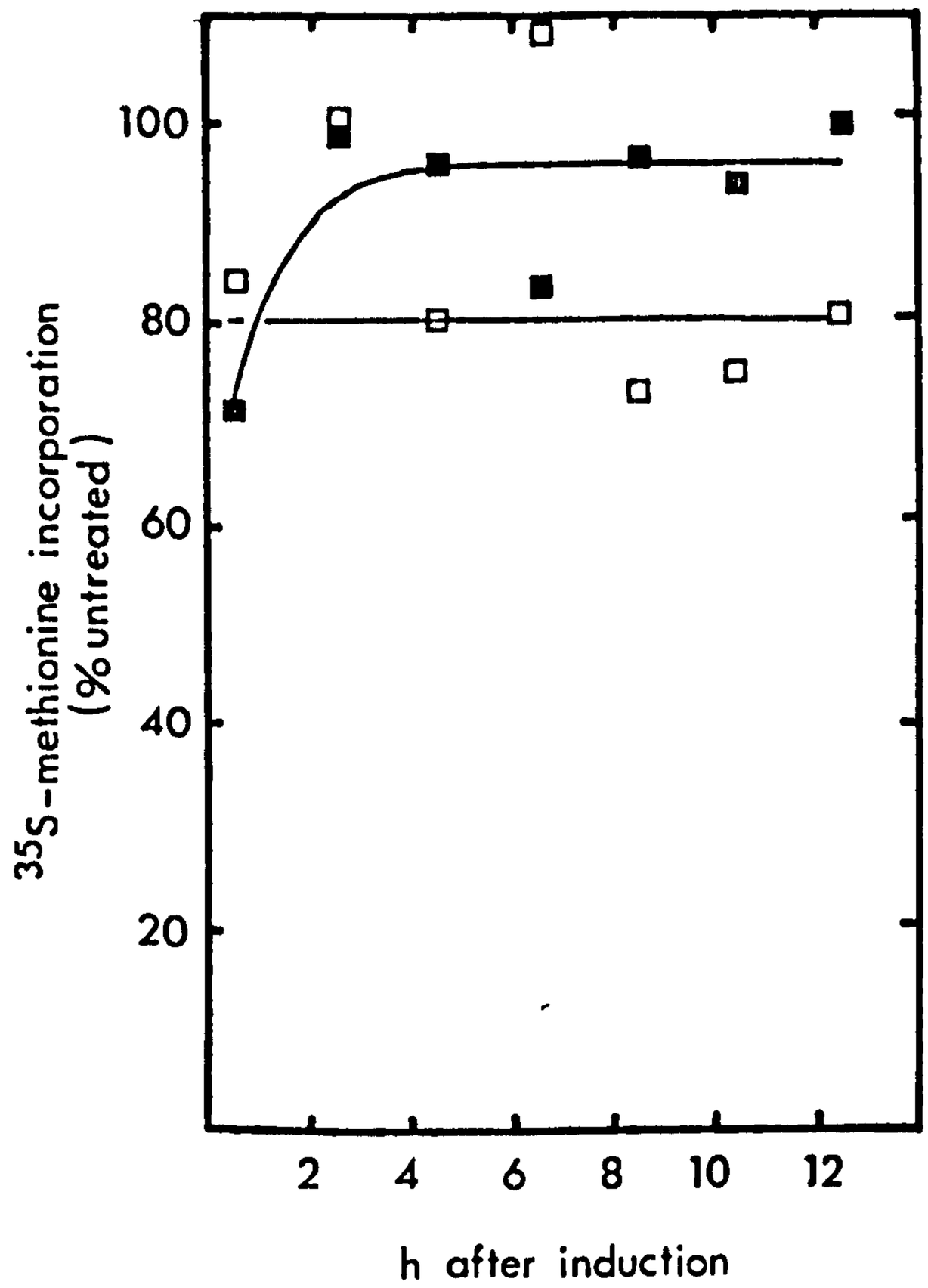


Fig.15. The incorporation of ^{35}S-methionine in treated cells. Cells were treated with butyrate (■) or BrdUrd (□) then induced. The incorporation of ^{35}S-methionine into TCA-insoluble material at various times after induction is expressed as a percentage of the incorporation into untreated, induced cells at each time point.

(average from 3 experiments)

after removal from butyrate and induction. In BrdUrd-treated cells ^{35}S-methionine incorporation was approximately 80% of that in untreated cells.

Increased efficiency of translation of IFNmRNA could occur as a result of reduced competition from other mRNA species in treated cells. For example, the inhibition of RNA synthesis by treatment with butyrate before induction could alter the mRNA population by depleting short half-life mRNAs. However, treatment had no effect on the distribution of free and ribosome bound IFNmRNA, approximately 80% of the IFNmRNA being associated with polysomes prepared from treated and untreated cells. Table 11 shows the amounts of IFNmRNA in RNA associated with polysomes which were extracted from butyrate-treated, BrdUrd-treated and untreated cells at 8 h after induction. The increase in amounts of IFNmRNA caused by treatment was similar in both polysome associated and total RNA, therefore the proportion of IFNmRNA available for translation was similar in treated and untreated cells. This is consistent with the observation that butyrate and BrdUrd produce very few detectable effects on the rates of translation of other proteins (see section 3.4), which suggests that the treatments do not significantly alter the amounts or rates of translation of other mRNAs. By exclusion, butyrate and BrdUrd must therefore increase the rate of elongation, termination or processing of IFN or increase the number of ribosomes on each IFNmRNA.

h) <u>The relationship between amounts of IFNmRNA and IFN yield</u>

To consider the original question of how the amounts of IFNmRNA present in induced Namalwa cells relate to IFN yield, the data shown in Figs. 11a and 11b together with other relevant data were analysed using a computer programme (Royal Society statistical package, GLIM) to plot amounts of IFNmRNA against IFN yield. When the data were

Table 11. The amount of IFNmRNA present in total RNA and polysomal RNA extracted from treated and untreated cells.

treatment	IFN (units/10oocytes)	
	total RNA	polysomal RNA
butyrate	112	100
BrdUrd	63	50
none	25	35

Cells were treated with 0.8 mM butyrate, 25ug/ml BrdUrd or maintenance medium then induced. At 8h after induction total RNA and polysomal RNA was prepared and microinjected into oocytes. The IFN translated was used to determine the relative amount of IFNmRNA present in each sample.
(single samples)

plotted collectively, the line of best fit produced a slope of 1.215 ± 0.267 with the deviance of points from the line being 22.65. Interestingly, the line intercepted the y (IFN yield) axis at around 2.0. It was rarely possible to detect IFNmRNA by translation in oocytes of RNA extracted from cells producing less than 2.0 Log_{10} units/10^6 cells of IFN, therefore this probably represented the lower limits of the oocyte translation assay. When the y intercept was fixed at 2.0 and the data for treated and untreated cells plotted separately, the deviance of points from the lines and standard dev iation of the slopes were considerably reduced. Fig. 16 shows this interpretation of the data. The line of best fit for data from untreated cells had a slope of 1.06 ± 0.149, with the deviance of points from the line being 4.5. The slope would indicate that in untreated cells, the increase in IFN yield was directly proportional to the increase in amounts of IFNmRNA. For treated cells, the line of best fit gave a steeper slope of 1.547 ± 0.064 with the deviance of points being 7.5. This was consistent with the observation that in treated cells the increase in IFN yield was not directly proportional to the increase in amounts of IFNmRNA, and favours the interpretation that butyrate and BrdUrd increase the efficiency of IFNmRNA translation.

j) <u>Discussion</u>

These results have shown, together with those of Morser et al., 1980, that butyrate and BrdUrd both caused a dose-dependant increase in the amounts of IFNmRNA and IFN yield induced in Namalwa cells. The treatments did not cause any changes in the time course of accumulation or decay of IFNmRNA, consistent with the fact that the time course of IFN production was the same in treated and untreated cells. Therefore the increase in amounts of IFNmRNA must result from an increased rate of synthesis. The most likely explanation would be increased

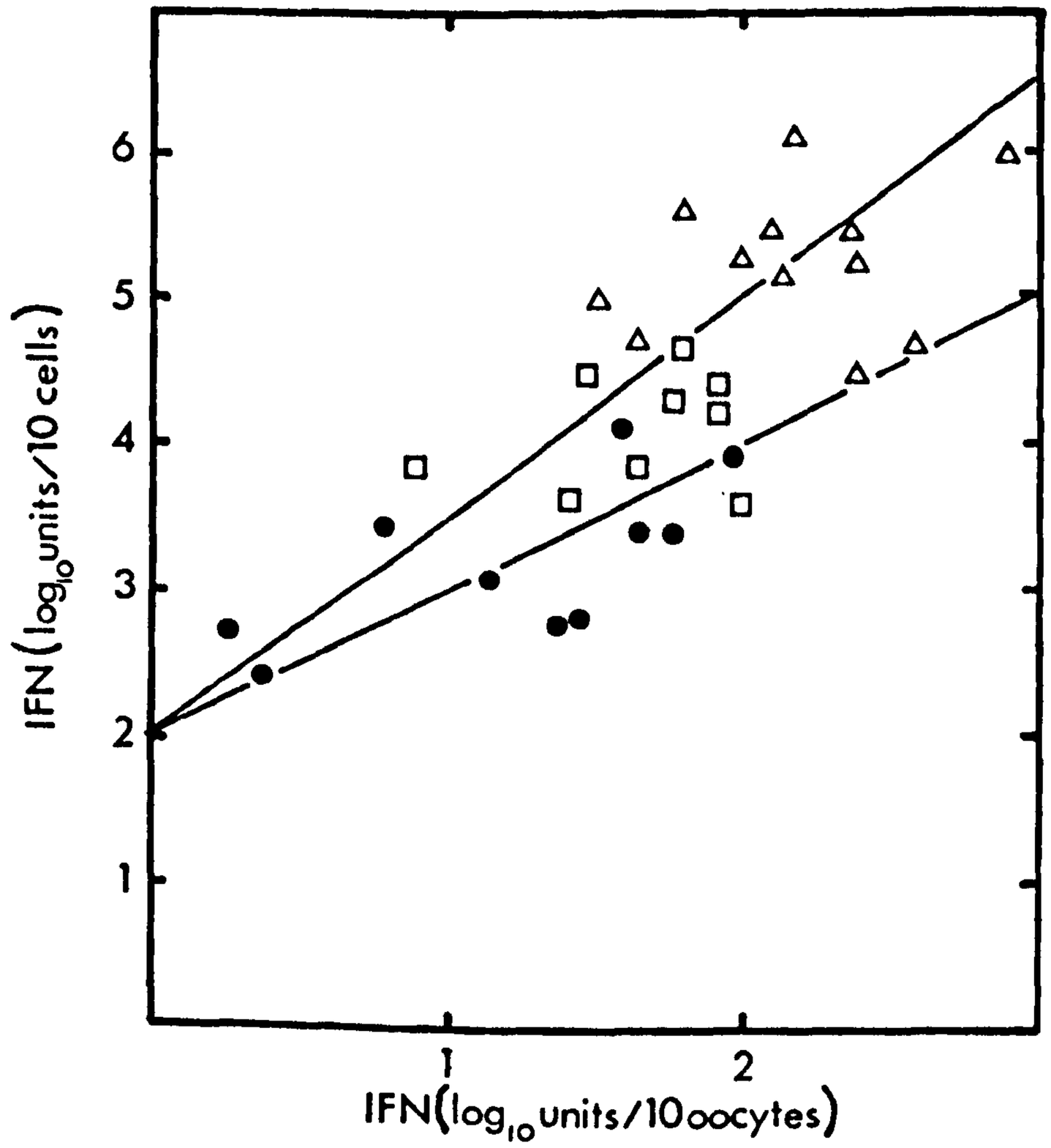


Fig.16. The relationship between IFN yield and amounts of IFNmRNA in butyrate-treated (△), BrdUrd-treated (□) and untreated cells (●). Data were plotted as described in the text.

transcription of the IFN genes, however these data cannot distinguish this from increased processing or export of transcripts from the nucleus. As in section 3.1, accurate measurements of rates of transcription and degradation, using pulse-labelled nascent RNA, would help to determine the effects of butyrate and BrdUrd on transcription and degradation of IFNmRNA.

An alternative explanation for the effect of butyrate on amounts of IFNmRNA and IFN yield would be that treatment increases the number of cells responding to IFN induction. However, data kindly provided by Dr D Barlow indicated that this does not occur. Using a single-cell assay designed to measure the number of cells producing IFN in a population of cells induced by NDV, it was found that 40% of both butyrate-treated and untreated Namalwa cells were making sufficient IFN to be detected in the assay. Therefore butyrate did not increase the amounts of IFNmRNA and IFN produced by the cells population by increasing the number of cells responding to induction.

In untreated cells there was a direct relationship between amounts of IFNmRNA and IFN yield, as might be expected. However, the data from treated cells have been interpreted to suggest that butyrate and BrdUrd not only increased the rate of synthesis of IFNmRNA, but also the rate of its translation, so that the amounts of IFNmRNA are only indirectly related to IFN yield. The reasons for this effect on translation are not known, but several observations indicate that it is a non-specific effect of butyrate and BrdUrd treatment which is unrelated to the increase in amounts of IFNmRNA.

i) The difference of 3-4 fold between the increases in IFNmRNA and IFN yield was the same for both treatments and not dose-dependant.

ii) It would appear that the condition of the cells before treatment was critical. Firstly, when the cells were held in maintenance medium

for 48 h before butyrate or BrdUrd treatment the increase in amounts of IFNmRNA equalled the increase in IFN yield. Secondly, in previous studies on the effects of butyrate on IFNmRNA and IFN synthesis (Morser et al., 1980), both were increased to the same extent. The only difference between the experiments reported here and those by Morser et al., 1980 would appear to be the passage conditions they used to grow cells before treatment. Cells were grown to a density of 2×10^6 cells/ml or more, whereas in this study cell density never exceeded 10^6 cells/ml and cells were routinely passaged 2 days before each experiment. Namalwa cell cultures exceeding 2×10^6 cells/ml are no longer growing exponentially, and similarly growth is arrested if cells are held in maintenance medium. This suggested that the metabolic status of cells was important in determining whether or not butyrate and BrdUrd treatment caused increased efficiency of translation of IFNmRNA. No further investigations were carried out since this was not a specific effect of butyrate or BrdUrd.

In conclusion, IFN synthesis is directly proportional to the amounts of IFNmRNA present in untreated, induced cells. Butyrate and BrdUrd treatments increase the rate of IFN synthesis by increasing the rate of IFNmRNA synthesis. The treatment conditions also caused an increase in the efficiency of IFNmRNA translation which contributed to the enhanced yields of IFN. The value of butyrate and BrdUrd as tools for studying the control of IFN synthesis is discussed in section 4.

Section 3.3 Analysis of Namalwa IFNmRNA

a) Introduction

The data in the previous two sections, describing the control of IFNmRNA synthesis and its stability in Sendai virus induced Namalwa cells, were obtained entirely by translation of the mRNA in oocytes. While this method was sufficient to determine the relative amounts of functional IFNmRNA, and hence the capacity of the cells to produce IFN, it has several important limitations. Firstly, the oocyte translation method considered all IFNmRNAs collectively and the proportions of IFN-α and IFN-β mRNA were not distinguished. The Namalwa cells used in these experiments did not produce detectable IFN-β activity (see section 2) although this cell line has been reported to produce both IFN-α and IFN-β mRNA and protein (see section 1.2). Therefore it was important to know whether IFN-β mRNA was induced in these cells and if so, to determine the reason for the failure to produce IFN-β activity. Secondly, the measurement of IFNmRNA by translation in oocytes is indirect, being dependant on both the translational activity of the mRNA and the specific activity of the translated protein. Consequently, changes in the functional amounts of IFNmRNA, either during the period of IFN production or after treatment of the cells with butyrate and BrdUrd, are not necessarily equivalent to changes in physical amounts of IFNmRNA. In addition information was required about changes in the composition of IFNmRNA, for example do butyrate and BrdUrd alter the relative proportions of IFN-α to IFN-β mRNA and likewise do they cause increased synthesis of the multiple IFN-α mRNA species individually or collectively? This information is important for understanding the control of IFN synthesis and also the action of butyrate and BrdUrd.

Hybridization of induced RNA with cloned IFNcDNA probes provides an alternative and complimentary method of identifying and quantitating IFNmRNA. The method allows each class of IFNmRNA to be characterised specifically since the lack of sequence homology between IFN-α and IFN-β prevents cross-hybridization and in addition it is dependant on the physical amounts of mRNA present. In this section the IFNmRNA induced in Namalwa cells is investigated by hybridization with IFN-α and IFN-β cDNA probes. The data are used in conjunction with measurements of IFNmRNA activity in oocytes to describe in more detail the expression of IFNmRNA in these cells

Unless otherwise stated, RNA from butyrate-treated cells was used because butyrate treatment increased the amounts of IFNmRNA available for investigation. This approach was based on the assumption, which will be validated in part g, that treatment did not alter the characteristics of the IFNmRNA induced.

b) <u>Characterisation of the Namalwa IFNmRNA</u>

The distinction between IFN-α and IFN-β mRNA classes by oocyte translation relies on measurement of the neutralisation of translated IFN activity by antisera raised to each class of IFN. Using this approach no IFN-β activity, and hence no IFN-β mRNA, could be distinguished by microinjection of oocytes with either total or poly(A)$^+$ RNA from induced Namalwa cells. (data not shown). Because of the problems caused by cross-reactivity of the antisera mentioned in section 2, this result did not eliminate the possible presence of small quantities of active IFN-β mRNA which could be masked by the translation of predominantly IFN-α.

In contrast, dot-blot hybridisation of RNA from Namalwa cells with ^{32}P-labelled IFN-α2 and IFN-β cDNA indicated that both classes of IFNmRNA were readily detectable after (But not before) induction

with Sendai virus. The size of the RNA hybridizing with these probes was estimated by fractionating poly(A)$^+$RNA from induced, butyrate-treated cells under fully denaturing conditions by electrophoresis through 1.5% agarose, 10mm methylmercury gels. The RNA was then transferred by blotting, to nitrocellulose filters and hybridized with ^{32}P-labelled IFN-α 2 cDNA in order to locate the IFN-α mRNA species present. Only an IFN-α 2 cDNA probe was used, therefore the stringency of the hybridization was reduced to allow for the lack of sequence homology, and ensure that all IFN-α mRNA species were detected (see section 1.2). Fig. 17, track 1 shows that the IFN-α mRNA species were represented by a heterogeneously sized population of molecules. Two broad bands could be distinguished around 1.25Kb and 1.4Kb in size. The average size of these bands (from 6 determinations) was 1.20$\pm$ 0.03Kb and 1.35 $\pm$ 0.03 kb. When the stringency of hybridization was increased the same size distribution was observed, although this reduced the degree of hybridization (judged by the intensity of the signal). Therefore the reduced stringency was not introducing artifactual results and Fig. 17, track 1 represented specific hybridization with IFN-α mRNA.

The size of the IFN-β mRNA present in the poly(A)$^+$RNA from induced cells was determined using the same filter. The IFN-α 2 cDNA probe was melted off and the filter rehybridized with labelled IFN-β cDNA using normal stringency conditions. Fig. 17, track 2 shows that a single band of IFN-β mRNA was detected with a size of 1.1Kb. The average size if this band was 1.05$\pm$ 0.03Kb (from 6 determinations) which was clearly distinct from the size of IFN-α mRNAs. Therefore since these sizes corresponded closely with the expected sizes of IFNmRNA species (see part h), it was concluded that both IFN-α and IFN-β mRNA were present in these Namalwa cells.

Fig.17. Blot-transfer hybridization analysis of RNA from induced cells. 20ug of poly(A)$^+$ RNA prepared from cells at 7h after induction were electrophoresed through an agarose-methyl-mercury gel and transferred to a nitrocellulose filter. The immobilised RNA was first hybridized with ^{32}P-labelled IFN-α cDNA (track 1). The probe was then melted off and the RNA rehybridized with ^{32}P-labelled IFN-β cDNA (track 2).

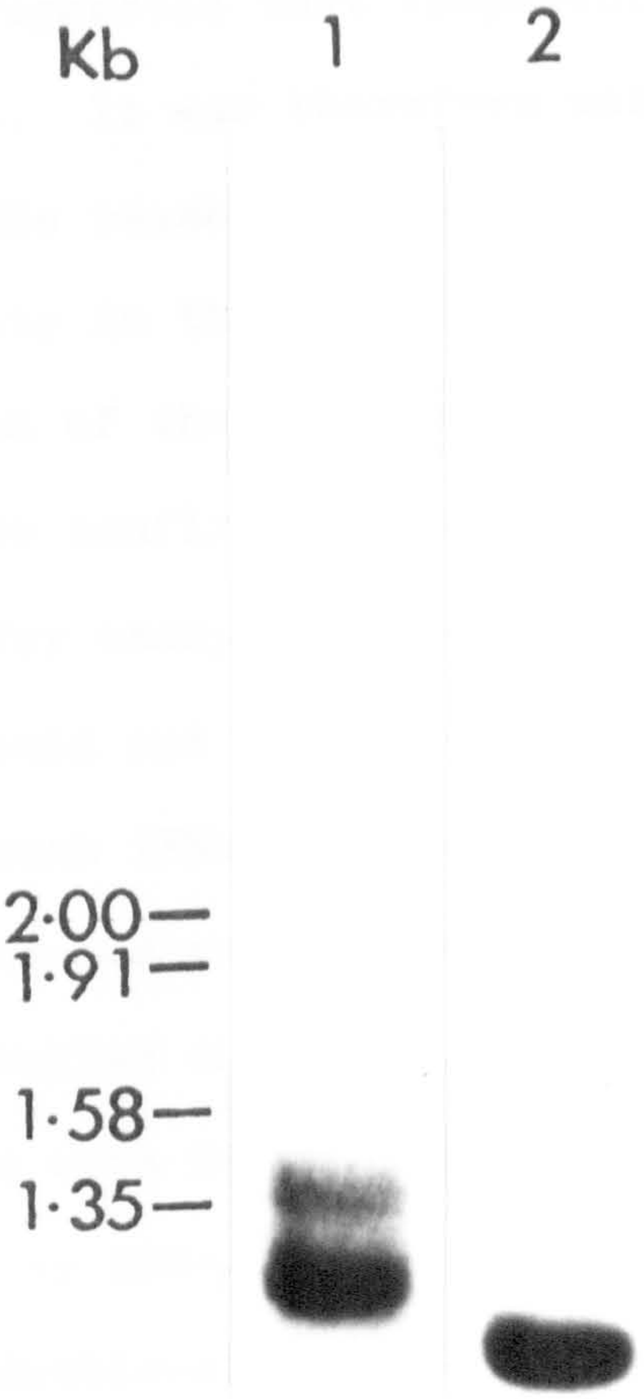


Fig.17

c) <u>The relative abundance of IFN-α and IFN-β mRNA</u>

Comparison of the amounts of probe hybridizing with dot-blots of RNA from induced cells suggested that comparable amounts of IFN-α and IFN-β mRNA were present. It was therefore unlikely that low levels of IFN-β mRNA were the reason for the failure to detect translation of IFN-β activity in the Namalwa cells or in oocytes. However, a direct comparison of the amounts of probe hybridizing to RNA was not sufficient to confirm the relative proportions of IFN-α and IFN-β mRNA. For example, possible differences in hybridization efficiency could not be discounted. In order to estimate the relative abundance of each IFNmRNA as a percentage of the total poly(A)$^+$ population in untreated cells, the dot-blot hybridizations were calibrated using unlabelled cDNA bound to nitrocellulose filter discs. Filters were loaded with 0-5ng of denatured double-stranded DNA containing the IFN-α 2 or IFN-β cDNA in a total of 5ug of carrier salmon sperm DNA. (Since double-stranded probe was to be used the double-stranded nature of the unlabelled DNA on the filters could be ignored). The DNA on the filters was hybridized with the appropriate labelled cDNA probe (using an approximate 10-fold excess of labelled complementary DNA), washed and then counted by Cerenkov emmision to quantitate the amount of probe hybridized. Fig. 18 shows that there was a linear relationship between DNA loaded and counts hybridized for both the IFN-α and IFN-β calibration. In order to estimate the abundance of IFN-α and IFN-β mRNA filters carrying 0-5ug of poly(A)$^+$RNA from untreated induced cells (in a total of 5ug, made up with carrier yeast RNA) were included in the hybridization together with the filters used to calibrate the assay. By comparing the amount of each probe which hybridized with the RNA in Fig. 19 and the calibration curves in Fig. 18, the relative proportions of IFN-α and IFN-β mRNA were calculated as follows. 5ug of poly(A)$^+$RNA

Fig.18. Dot-blot calibration. From 0-5 ng of (a) IFN-α 2cDNA and (b) IFN-β cDNA were dot-blotted onto nitrocellulose filters with 5ug of carrier DNA. The filters were then hybridized with their respective ^{32}P-labelled cDNAs, and then counted by Cerenkov emmission.

(duplicate samples from a single experiment)

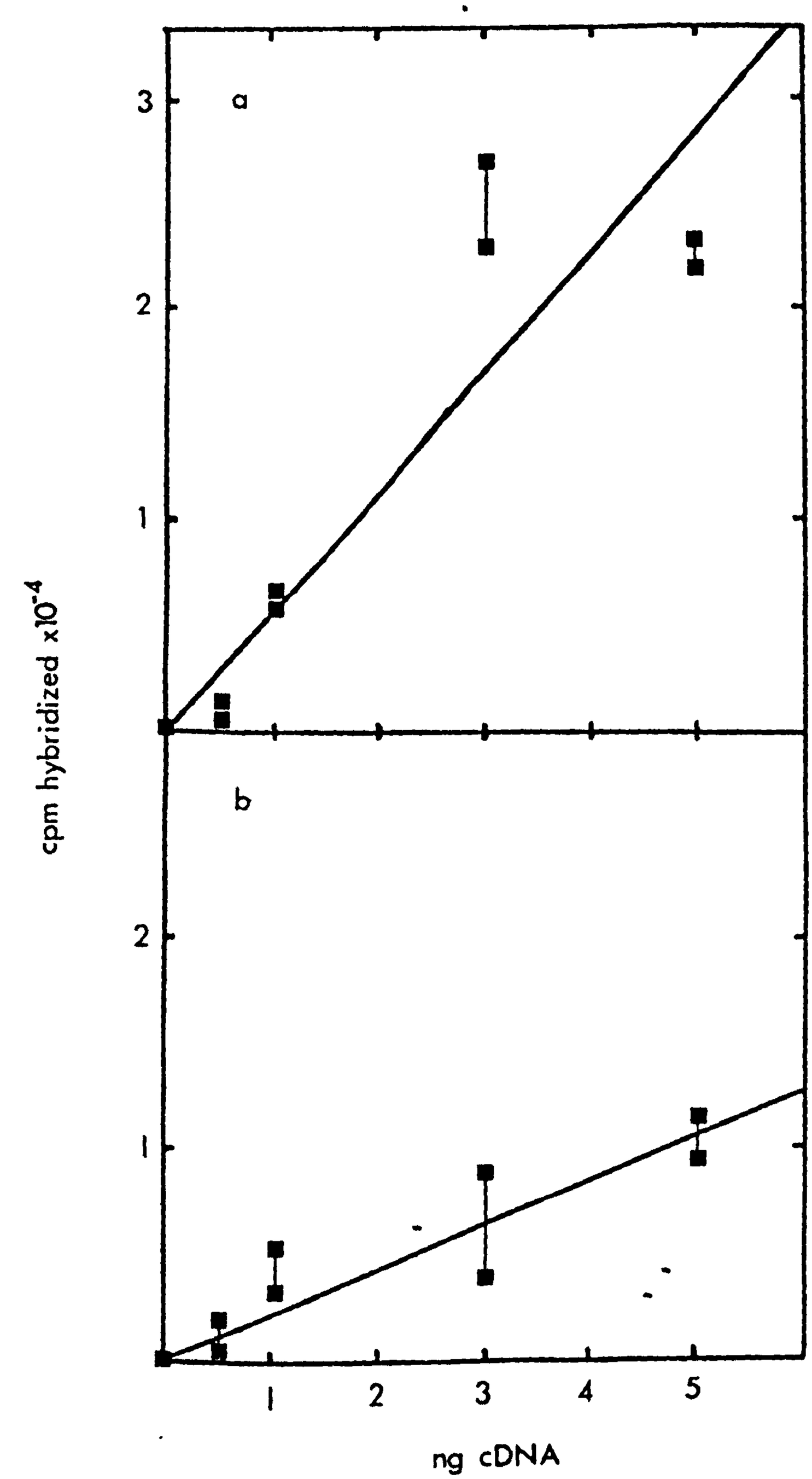


Fig.18

Fig.19. Dot-blot calibration. From 0-5ug of poly(A)$^+$ RNA from untreated, induced cells wasdot-blotted onto nitrocellulose filters and hybridized with ^{32}P-labelled IFN-α 2cDNA (a) or IFN-β cDNA (b). The amount of probe hybridized was estimated by Cerenkov emmission.

(duplicate samples from a single experiment)

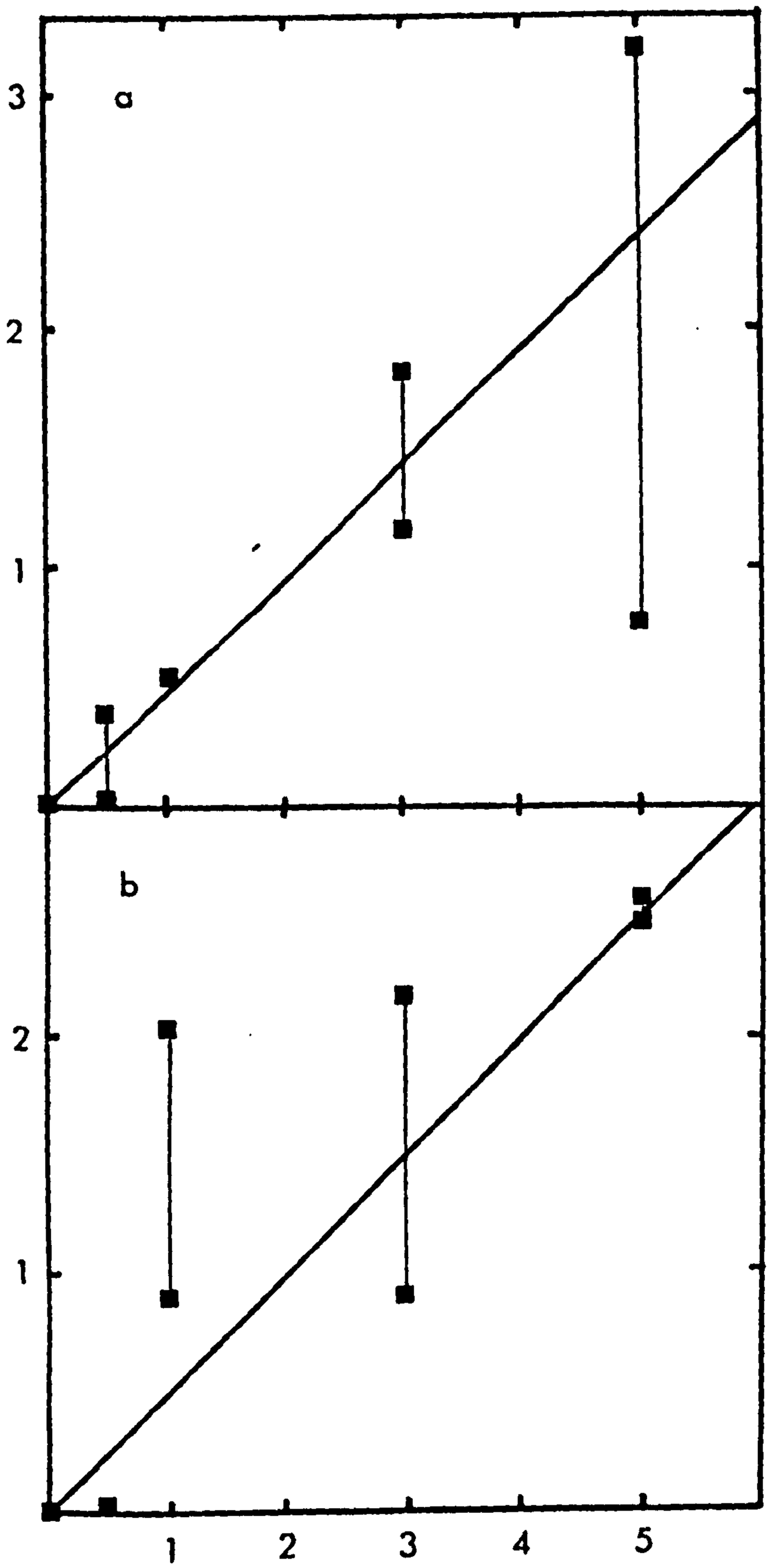


Fig.19

contained the equivalent of 0.5ng of IFN-α cDNA and 1.2ng of IFN-β cDNA. These values were corrected for the difference in size between the DNA fragments containing the cDNAs and their respective mRNAs. The size of the Msp fragment containing the IFN-α2 cDNA was 1.18Kb (see section 2) whereas the average size of IFN-α mRNA was 1.27Kb (Fig. 17, trackl), therefore lng of DNA was equivalent to 1.08ng of mRNA. Similarly, the IFN-β cDNA was contained in a 1.21Kb fragment (see section 2) while IFN-β mRNA was 1.05Kb in size (See Fig. 17, track 2) therefore lng of DNA was equivalent to 0.86ng of mRNA. From this it was estimated that IFN-α mRNA represented 0.01% and IFN-β mRNA 0.02% of the poly(A)$^+$RNA in this sample from untreated cells. On a molar basis the IFNmRNA was therefore composed of approximately 29% IFN-α and 71% IFN-βmRNA. Almost identical results were obtained when the calibration was repeated using different preparations of cDNA. Similar values were indicated by dot-blot hybridizations using over 20 other RNA samples.

These results indicated that sufficient IFN-β mRNA should be present in induced cells for its translation to be detected, since by comparison, the translation of IFN-α mRNA was readily detected. This suggested that the IFN-β mRNA was in some way defective. Two explanations for the failure to detect IFN-β activity in either induced cell cultures or oocytes microinjected with RNA from induced cells were i) the IFN-β mRNA is not translated or translated with a very low efficiency relative to IFN-α mRNA or ii) the IFN-β mRNA codes for an inactive protein.

Although the method used only provides an approximation of the relative abundance of each class of IFNmRNA, the values obtained for IFN-α mRNA compare well with other data which are discussed later in section h.

d) <u>The activity of fractionated IFNmRNA</u>

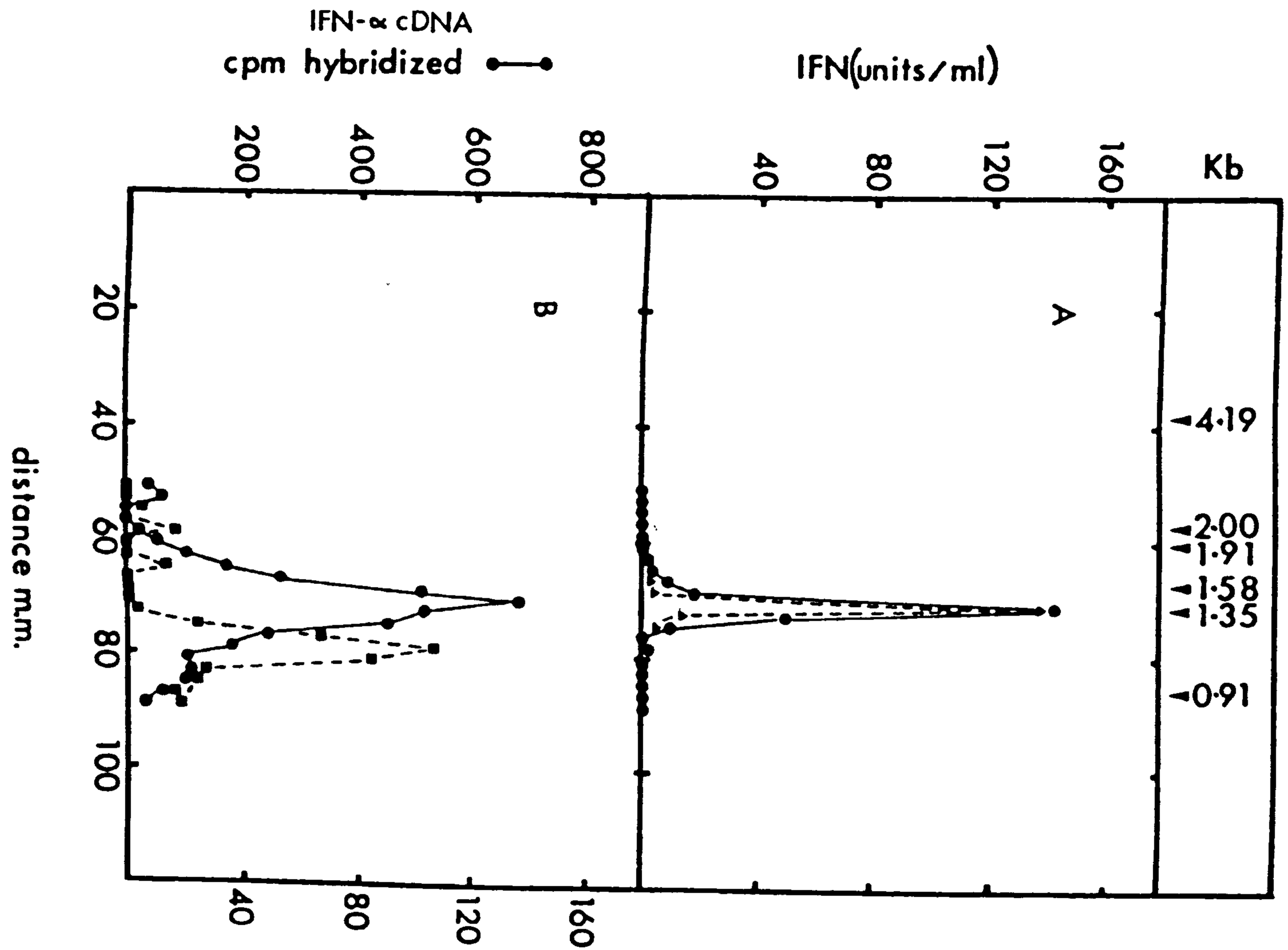
The characterisation of Namalwa IFNmRNA was extended by investigating the translational activity of the agarose gel fractionated RNA detected by hybridization with IFNcDNA probes. The reasons for this comparison were threefold; i) to determine whether the size distribution of IFN-α mRNA which hybridizes with IFN-α cDNA (See Fig. 17, track 1) also represents functional mRNA, ii) to confirm that no IFN-β activity is translated from the IFN-β mRNA detected by hybridization with IFN-β cDNA (in the absence of IFN-α activity which could mask small amounts of IFN-β) and iii) to ensure that no functional IFNmRNA (such as the large IFN-α mRNAs reported by Sagar et al., 1981) is produced other than the mRNAs identified by hybridization with IFNcDNA probes. To obtain the information, poly(A)$^+$RNA from induced, butyrate-treated cells was fractionated by methylmercury agarose gel electrophoresis as in Fig. 17. The gel was then cut into 2mm slices and the RNA eluted. An aliquot of the RNA from each gel slice was microinjected into oocytes in order to determine the position of active IFNmRNA. Fig. 20A shows the IFN activity translated from this RNA assayed in both bovine and human cells. For comparison, further aliquots of the same RNA samples were also analysed by dot-blot hybridization with either IFN-α 2cDNA or IFN-β cDNA probes, and Fig. 20B shows the location of gel slices containing IFN-α and IFN-β mRNA. From this it was apparent that active IFNmRNA was only detected in gel slices containing RNA 1.35Kb in size, which coincided with the region of the gel containing hybridizable IFN-α mRNA. No activity was detected by translation of RNA from other regions of the gel including RNA around 1.1Kb in size which hybridized with IFN-β cDNA.

Bovine cells are only sensitive to IFN-α, hence if any IFN-β activity were present it would only be detected by assay in human

Fig.20. Characterisation of fractionated IFNmRNA by oocyte translation and dot-blot hybridization. 20ug of poly(A)$^+$ RNA was electrophoresed through an agarose methylmercury gel.

(A) RNA was extracted from excised gel slices and microinjected into oocytes. The translated IFN was assayed in both bovine EBTr cells (●—●) and human GM2767 cells (▲--▲).

(B) Aliquots of the same RNA were dot-blotted onto nitrocellulose and hybridized with ^{32}P-labelled IFN-α 2cDNA (●—●) and IFN-β cDNA (■--■).


IFN-α cDNA
cpm hybridized
IFN(units/ml)
200
400
600
800
40
80
120
160
Kb
B
A
20
40
60
80
100
distance m.m.
4·19
2·00
1·91
1·58
1·35
0·91
40
80
120
160
IFN-β cDNA
cpm hybridized

cells and would result in a different profile of activity compared to assay in bovine cells. However, the distribution of functional IFNmRNA was identical when the translated IFN activity was assayed in either cell type. Furthermore, none of this IFN activity was neutralised by anti-IFN-β serum. The IFN assay using human GM2767 cells, as it was performed in this experiment, was capable of detecting as little as 0.4 units of IFN per ml. In the experiment shown in Fig. 20 this would mean that if active IFN-β were present it represented less than 0.25% of the total IFN activity translated by the RNA eluted from the gel. No active IFN-β mRNA could be detected by a similar analysis of RNA from untreated cells either (data not shown), therefore these results were not due to an effect of butyrate treatment.

The RNA extracted from agarose gel slices contained only partially purified IFNmRNA, since other RNAs of similar size would comigrate to the same position on the gel. To eliminate the possibility that these RNAs interfered with translation of IFN-β mRNA, a control experiment was set up to purify the IFNmRNAs. Poly $(A)^+$RNA from induced , butyrate-treated cells was hybridized with either IFN-α 2cDNA or IFN-β cDNA which had been immobilised on nitrocellulose filters. The hybridized RNA was then eluted and translated by microinjection into oocytes. Table 12 shows data kindly provided by Dr Morser which demonstrates that the RNA released from IFN-α 2cDNA translated to produce IFN activity which was characterised as IFN-α by its high ratio of activity in bovine compared to human cells. This activity was also neutralised by anti-IFN-α serum (data not shown). The RNA released from IFN-β cDNA did not translate to give any IFN activity. As a check, RNA from NDV-induced MG63 cells (which produce both IFN-α and IFN-β) was also hybridized with both IFNcDNAs and clearly both IFN-α and IFN-β mRNA could be detected in RNA released

Table 12. Hybrid-release translation of IFNmRNA from induced Namalwa and MG63 cells.

RNA	cDNA	IFN (log_{10}units/ml) EBTr	IFN (log_{10}units/ml) HFF
Namalwa	α	2.1	1.2
MG63	α	1.1	< 0.3
Namalwa	β	< 0.3	< 0.3
MG63	β	< 0.3	1.6

Poly(A)$^+$ RNA from induced Namalwa and MG63 cells was hybridized with either IFN-α2 or IFN-βcDNA which had been immobilised on nitrocellulose filters. Hybridized RNA was removed from the filters and microinjected into oocytes. The IFN translated was assayed in both bovine (EBTr) and human (HFF) cells. (single samples from a representative experiment)

from the respective cDNAs.

Together these results indicated that the IFN-β mRNA induced in these Namalwa cells was not functional. In addition, the size distribution of functional IFN-α mRNA was identical with that of the IFN-α mRNA detected by hybridization. This did not mean that all of the hybridizable IFN-α mRNA was functional, but it did confirm that no IFNmRNA could be detected other than that which hybridized with IFN-α 2 and IFN-β cDNA probes.

e) <u>The distribution of IFNmRNA</u>

In order to obtain more information about the non-functional IFN-β mRNA its cytoplasmic distribution was compared with that of IFN-α mRNA. Measurements of functional IFNmRNA by translation in oocytes indicated that it was present exclusively in the poly(A)$^+$ fraction of cytoplasmic RNA, and that approximately 80% was associated with polysomes (and presumably involved in translation), (see section 3.2). Non-functional IFNmRNA could be present in the poly(A)$^-$RNA and possibly not be associated with polysomes. Therefore the physical distribution of IFN-α and IFN-β mRNA was compared by preparing polysome-associated and non-polysome-associated RNA from butyrate-treated, induced cells. Poly(A)$^+$ and poly(A)$^-$RNA was selected from each fraction and hybridized with either IFN-α or IFN-β cDNA probes. Table 13a shows that neither IFN-α or IFN-β sequences could be detected by hybridization with poly(A)$^-$RNA, therefore this fraction does not contain either active or inactive IFNmRNA. Hybridization with their respective probes showed that approximately 66% of the IFN-α mRNA and 76% of the IFN-β mRNA was in the poly(A)$^+$RNA associated with polysomes. Therefore not only was the relative distribution of each class of IFNmRNA similar, but it corresponded closely with the value of 80% obtained by

Table 13. The distribution of IFN-α and IFN-β mRNA in induced cells in the presence and absence of puromycin.

(a)

RNA sample		cDNA hybridized: IFN-α cpm	(%)	IFN-β cpm	(%)
polysomal pellet	poly(A)$^+$	2880	(66)	1069	(76)
free cytoplasmic	poly(A)$^+$	1434		330	
polysomal pellet	poly(A)$^-$	0		0	
free cytoplasmic	poly(A)$^-$	0		0	

(b)

pellet	poly(A)$^+$	446	(24)	136	(10)
free cytoplasmic	poly(A)$^+$	1377		288	
pellet	poly(A)$^-$	0		0	
free cytoplasmic	poly(A)$^-$	0		0	

(a) 5ug of both poly(A)$^+$ and poly(A)$^-$ RNA prepared from polysome associated and free cytoplasmic RNA were dot-blotted onto nitrocellulose filters and hybridized with IFN-α 2cDNA. The probe was then melted off and the RNA rehybridized with IFN-β cDNA. The amount of probe hybridized was estimated by Cerenkov emission.

(b) Samples were prepared and analysed as in (a) except that the RNA was prepared from cells which had been treated with puromycin (100 ug/ml) for 5 min.

(average of duplicate samples from a representative experiment)

measuring polysome associated IFNmRNA activity. It was possible that some of the mRNA pelleting with the polysomes was in fact associated with RNP particles, since the method of preparation depended on sedimentation of post-nuclear supernatants through a discontinuous sucrose gradient designed to optimize polysome yield (see section 2). To overcome this criticism a portion of the cells used to prepare polysomes was treated with puromycin 100ug/ml before preparing polysomes. This treatment disrupts the polysome complex by causing premature termination of polypeptide synthesis and detachment of the mRNA from ribosomes. Consequently, any RNA pelleting in the polysome preparation should not in fact be associated with polysomes but more likely RNP. Table 13b shows the distribution of IFN-α and IFN-β mRNA in this control experiment. Clearly the amounts of each IFNmRNA in the pellet were significantly reduced, following puromycin treatment of the cells. The small amounts remaining could be accounted for either by RNP or by small quantities of polysomes which remained, since the period of puromycin treatment was not sufficient to ensure complete dissociation. A longer exposure to puromycin would have meant that the RNA extracted in this experiment could no longer be compared with RNA extracted from polysomes, for example it could not be assumed that the stability of the mRNA or the integrity of its poly(A) tract would remain unaltered once removed from ribosomes. Nonetheless this result showed that most of the IFNmRNA pelleted during the preparation of polysomes was in fact associated with polysomes and not RNP.

The IFN-β mRNA was therefore a poly(A)$^+$mRNA which was specifically associated with polysomes to the same extent as the functional IFN-α mRNA. Consequently the absence of translated IFN-β activity was not due to a defect in the efficiency of IFN-β mRNA

ribosome binding.

f) <u>The Coordinate control of IFN-α and IFN-β mRNA synthesis and degradation</u>

Having established the time course of accumulation and decay of functional IFNmRNA in Section 3.2, it remained to determine firstly, whether physical amounts of IFNmRNA were regulated in the same way, and secondly, whether IFN-α and IFN-β mRNAs were coordinately controlled. For this purpose, total RNA extracted from butyrate-treated cells at various times after induction, was dot-blotted onto nitrocellulose filters then hybridized with either ^{32}P-labelled IFN-α 2cDNA or IFN-β cDNA. (The RNA samples were in fact aliquots of the RNA used in Section 3.2, Fig. 14b) Fig. 21 shows that the time course of accumulation and degradation of IFN-α and IFN-β mRNAs was identical. The amounts of both mRNAs reached a maximum at 9 hours after induction then declined as in Fig. 14b. Therefore both IFN-α and IFN-β mRNAs are induced and regulated coordinately.

The accumulation and decay of functional IFNmRNA therefore corresponds with similar changes in the physical amounts of IFNmRNA in the cells, indicating that the shut-off of IFN synthesis is accompanied by the degradation and not merely inactivation of its mRNA. However, the decline in amounts of functional IFNmRNA (Fig. 14b) slightly preceded the loss of hybridizable IFNmRNA (Fig. 21) as might be expected if the mRNA became non-functional while remaining sufficiently intact to be detected by the probe. The amounts of full-length IFNmRNA could have been measured by hybridization to fractionated RNA, in order to confirm this possibility, however the data in Fig. 16 were sufficient to conclude that degradation of IFNmRNA occurred on a similar time scale as its loss of translational activity.

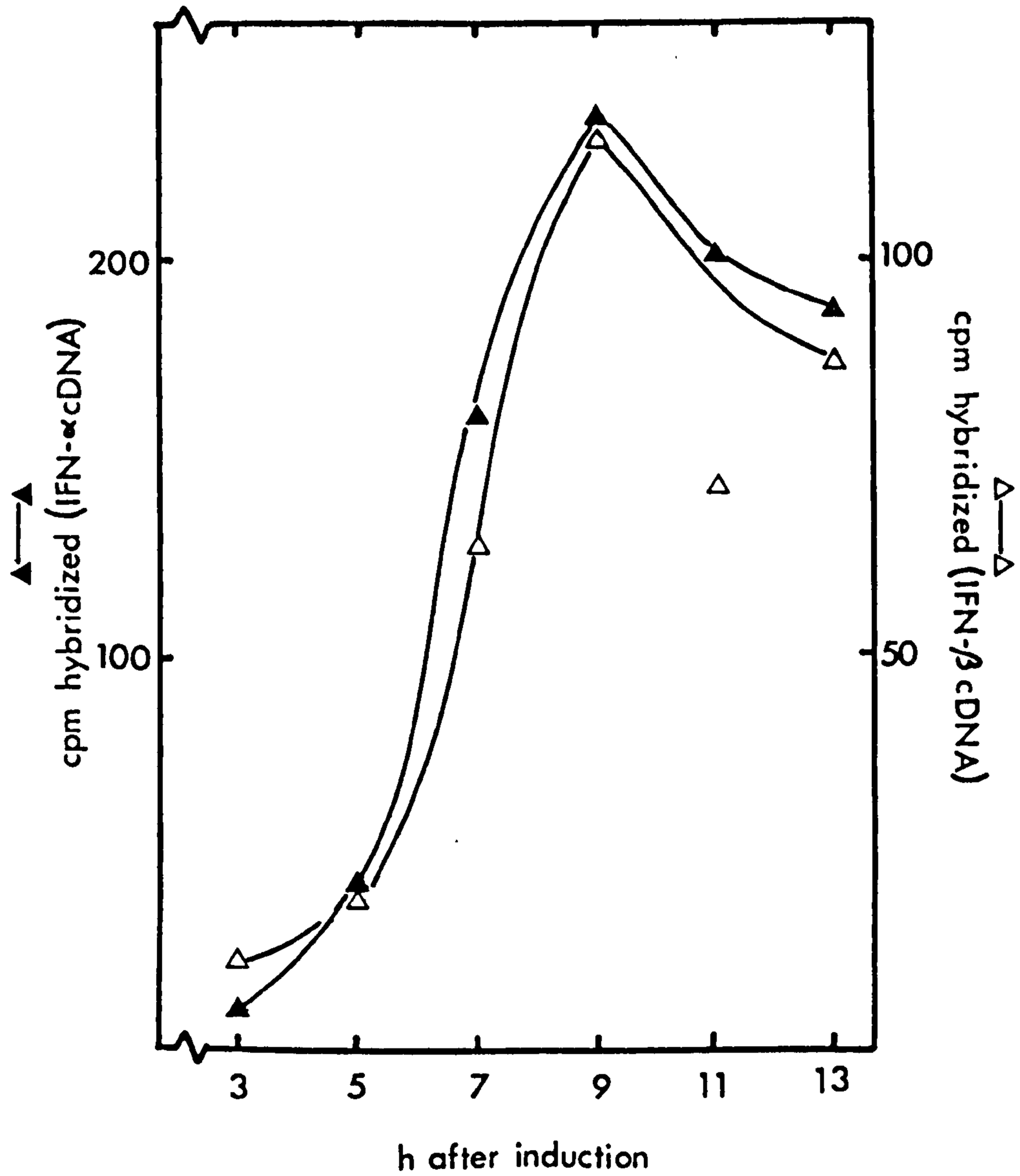


Fig.21. The coordinate control of IFN-∝ and IFN-β mRNA levels.

20ug of total RNA extracted from butyrate-treated cells at various times after induction were dot-blotted onto nitrocellulose filters. The RNA was then hybridized with either ^{32}P-labelled IFN-∝ 2cDNA (▲) or IFN-β cDNA (△).

(average of duplicate samples from a representativeexperiment)

It was shown in section 3.1 that by reducing the incubation temperature of cells to 28° at 7h after induction, IFNmRNA stability was increased thereby delaying the shut-off of IFN synthesis and increasing the overall yield of IFN. The conclusions relating to the effect of temperature on IFNmRNA stability (measured by oocyte translation of RNA extracted from cells at various times after induction) were confirmed by dot-blot hybridization of aliquots of the same RNA samples used in Fig. 10b with IFN-∝ 2cDNA probe. Fig. 22 clearly shows that the disappearance of IFN-∝ mRNA is delayed in cells incubated at 28°, and confirms that the shut-off of IFN synthesis and decay of functional IFNmRNA (see Fig. 10b) is accompanied by the degradation and loss of IFNmRNA from the cells.

g) <u>The effects of butyrate and BrdUrd on IFNmRNA synthesis</u>

In section 3.2 treatment of cells with butyrate or BrdUrd before induction was shown to increase the synthesis of translatable IFNmRNA. Although the characteristics of the IFN produced by cells were not affected by these treatments it was still necessary to compare the characteristics of the IFNmRNA produced by treated and untreated cells and to confirm that increased physical amounts of IFNmRNA were synthesised. This information was required to describe more fully the effects of butyrate and BrdUrd on IFN synthesis. For these reasons the size distribution and differences in the physical and functional amounts of IFNmRNA were compared in treated and untreated cells.

Poly(A)$^+$RNA from induced, butyrate-treated, BrdUrd-treated and untreated cells was electrophoresed through an agarose methyl-mercury gel, transferred to nitrocellulose filter and then hybridized with IFN-∝ 2cDNA as in Fig. 17. Fig. 23 shows that the IFN-∝ mRNA detected has the same heterogeneous size distribution in RNA from

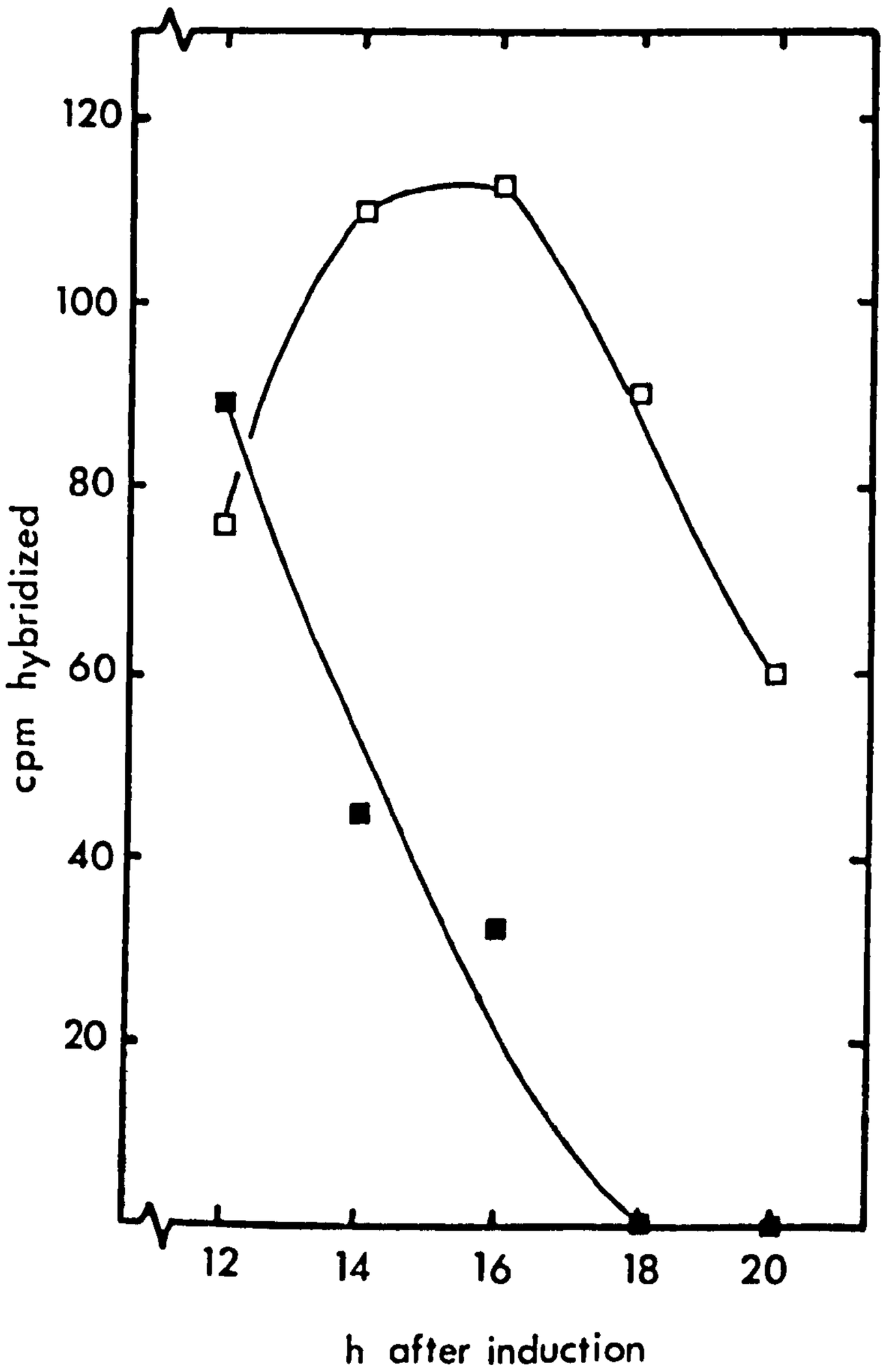


Fig.22. The effect of reduced incubation temperature on IFNmRNA levels during the shut-off of IFN synthesis. Total RNA was extracted from cells incubated at 37° (■) or at 28° from 7h after induction (□). 20ug of the RNA was dot-blotted onto nitrocellulose and hybridized with ^{32}P-labelled IFN-α 2cDNA.

(average of duplicate samples from a single experiment)

Fig.23. Blot-tranfer hybridization analysis of IFN-α mRNA from induced, treated and untreated cells. 20ug of poly(A)$^+$ RNA extracted at 7h after induction from (1) butyrate-treated, (2) untreated and (3) BrdUrd-treated cells was electrophoresed through an agarose-methylmercury gel and transferred to a nitrocellulose filter. The immobilised RNA was then hybridized with ^{32}P-labelled IFN-α cDNA. (4) shows a shorter autoradiograph exposure of (3).

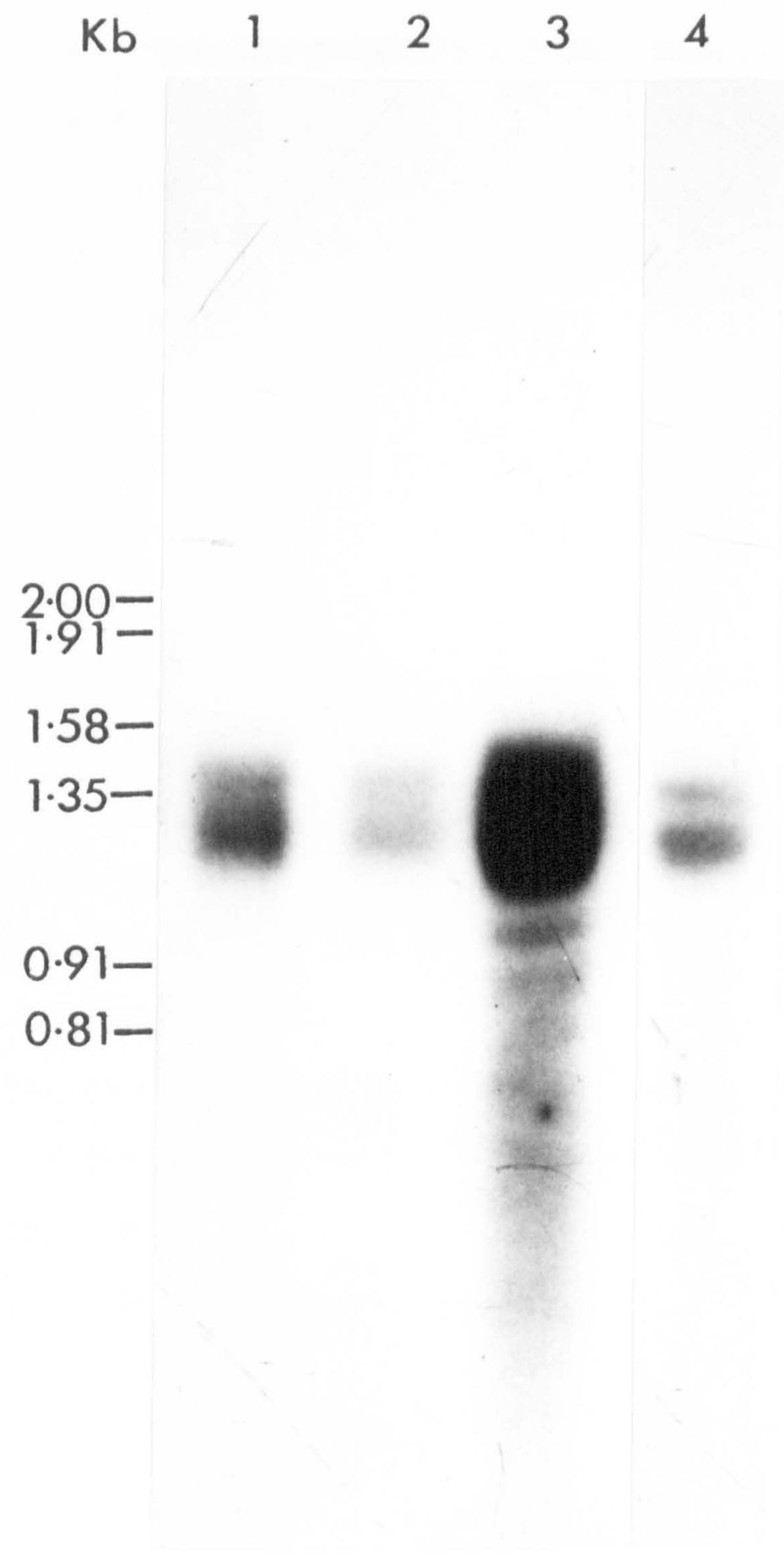


Fig.23

both treated and untreated cells, with two broad bands being resolved around 1.2 Kb and 1.35 Kb in size. No hybridization was detected with RNA larger than approximately 1.5 Kb. Fig. 23, track 4 shows a shorter autoradiograph exposure of track 3, confirming that the RNA was identical in size. The hybridization with smaller RNA in track 3 was most probably due to degraded IFN-α mRNA, and was not unexpected since the mRNA is rapidly turned over (see section 3.1).

Fig. 24 shows densitometric scans of the autoradiograph shown in Fig. 23 tracks 1-3. By measuring the area beneath each peak, the increase in hybridization was found to be 18-fold for RNA from butyrate-treated cells and 3-fold for RNA from BrdUrd-treated cells relative to RNA from untreated cells.

Identical samples to those used in Fig. 23 were electrophoresed on the same gel, and the gel tracks were cut into 2mm slices. An aliquot of the RNA extracted from each gel slice was microinjected into oócytes and the translation products assayed for IFN-α activity in bovine cells. Fig. 25 shows that the profile of IFN-α mRNA activity was identical for treated and untreated cells, with a single peak occurring around 1.35 Kb in size. No IFN activity was detected elsewhere on the gel by assay in human or bovine cells and none of the IFN activity was neutralised by anti-IFN-β serum, as in Fig. 20a, therefore no active IFN-β mRNA could be detected. The increase in active IFN-α mRNA, calculated by integrating each peak, was 10-fold for butyrate-treatment and 4-fold for BrdUrd treatment which closely compared with the values obtained for the unfractionated RNA (10-fold for butyrate and 1.5-fold for BrdUrd treatment). Therefore the increase in amounts of active IFNmRNA in butyrate and BrdUrd-treated cells could be accounted for by RNA of this size, and it was unlikely that IFNmRNA of another size had gone undetected (for example, due to

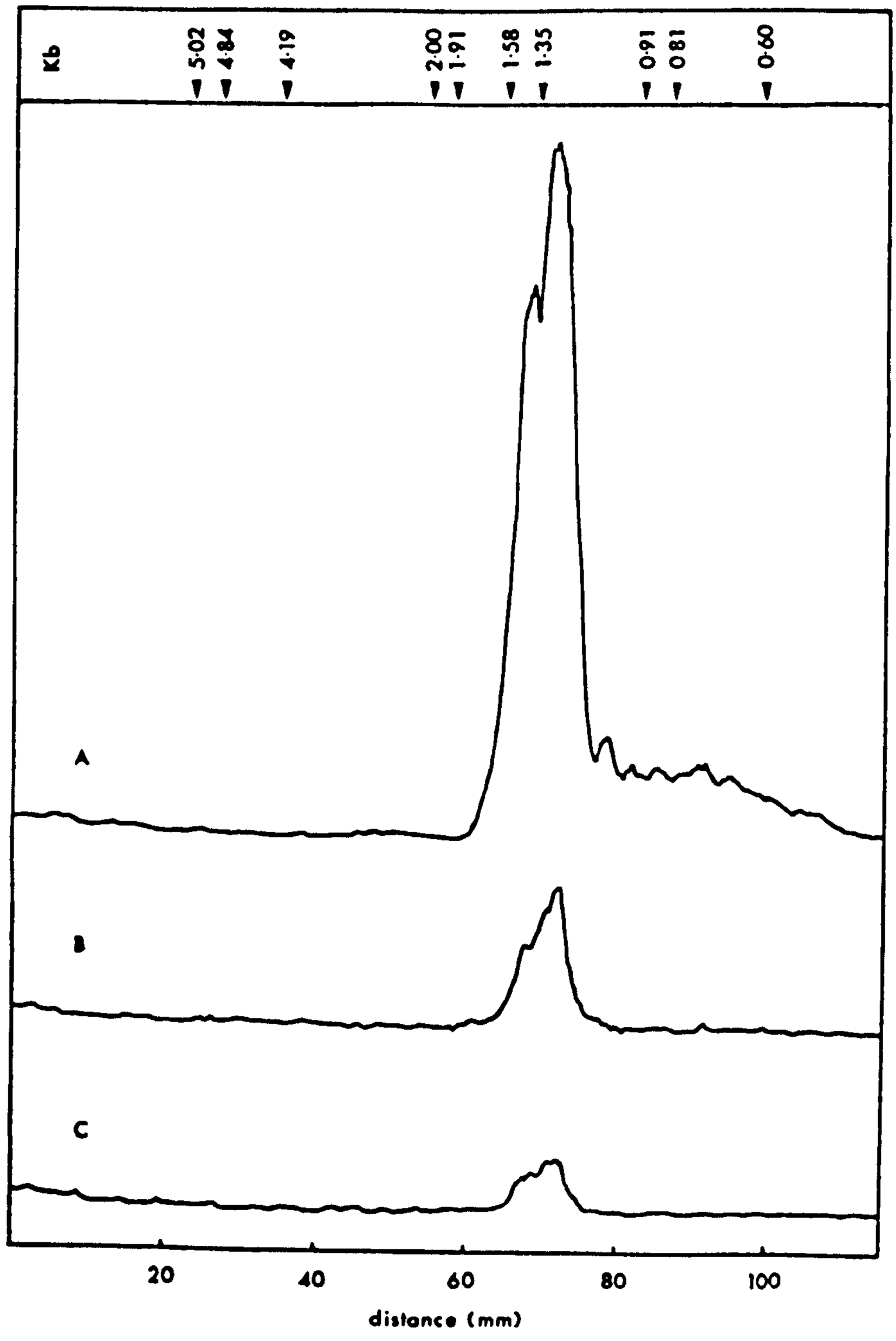
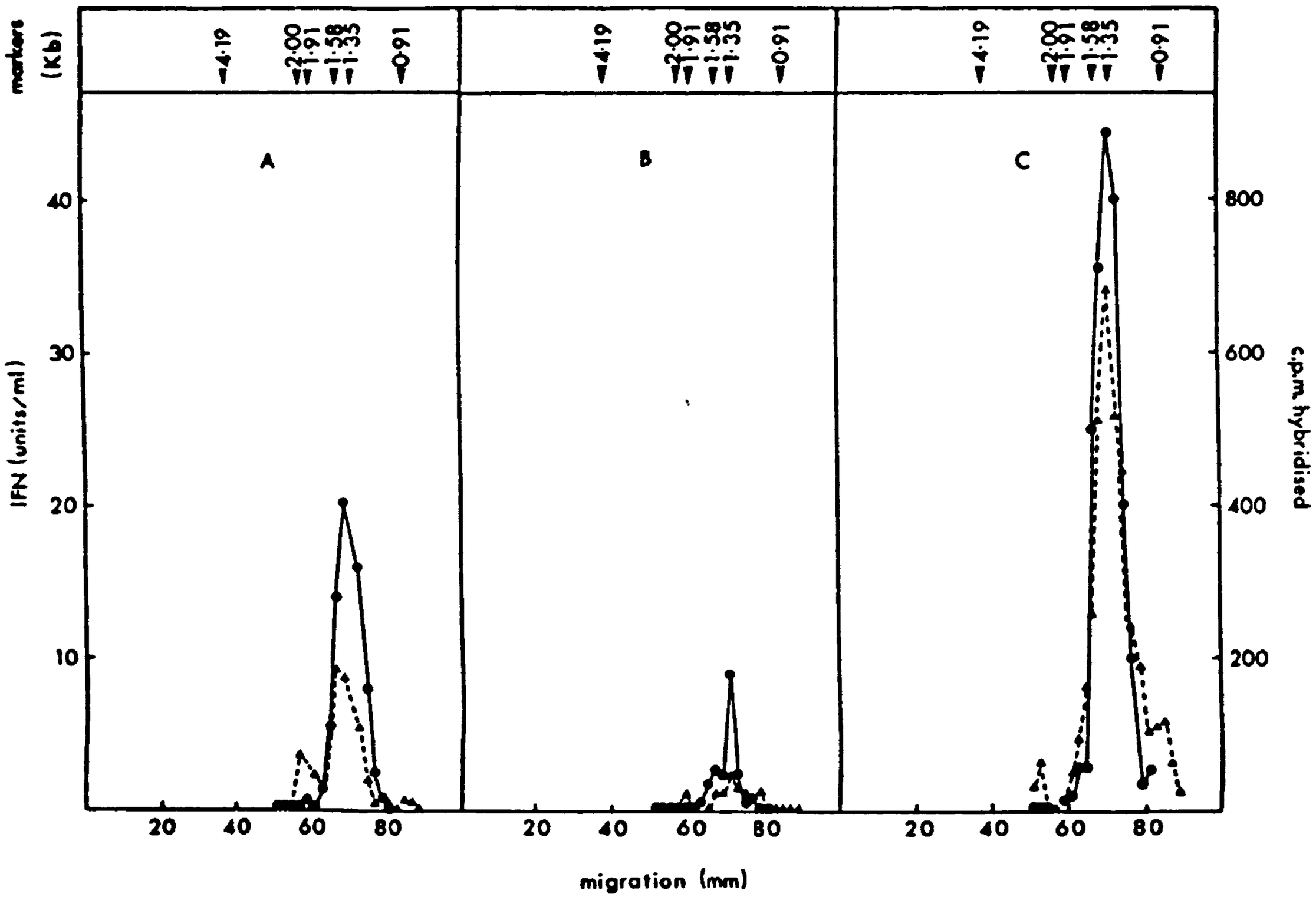


Fig.24. Densitometric scans of the autoradiograph shown in Fig.23. (A) RNA from butyrate-treated cells, (B) RNA from BrdUrd-treated cells and (C) RNA from untreated cells.

Fig.25. Characterisation of the IFNmRNA in treated and untreated cells.

20ug of poly(A)$^+$ RNA from (A) BrdUrd-treated , (B) untreated and (C) butyrate-treated cells were electrophoresed through a methylmercury agarose gel. RNA was extracted from excised gel slices and analysed both by translation in oocytes (●—●) and by dot-blot hybridization (▲--▲)with ^{32}P-labelled IFN-α2 cDNA.


markers (Kb)
4·19
2·00
1·91
1·58
1·35
0·91
A
B
C
IFN (units/ml)
c.p.m. hybridised
migration (mm)
10
20
30
40
200
400
600
800
20
40
60
80

inefficient elution from the gel or inactivation).

Another aliquot of the same RNA samples used for microinjection into oocytes was analysed by dot-blot hybridization in order to correlate the location of active and hybridizable IFNmRNA. Fig. 25 shows the amount of IFN-α 2cDNA hybridizing to each sample, and clearly the size distribution of IFN-α mRNA was identical for both treated and untreated cells. The increase in amounts of hybridizable IFN-α mRNA (measured by integrating each peak) was 16-fold for RNA from butyrate-treated cells and 4-fold for RNA from BrdUrd-treated cells. Although the dot-blot analysis did not distinguish the two bands of hybridization seen in Fig. 17a (as might be expected, since gel slicing provides poor resolution), it indicated that the physical distribution of IFN-α mRNA coincided with the distribution of active IFN-α mRNA. This does not indicate that all of the IFN-α mRNA detected by hybridization is active, since neither technique measures absolute amounts of mRNA.

Together these results suggested that butyrate and BrdUrd treatments caused a proportionate increase in all of the IFN-α mRNA species transcribed in untreated cells, and did not cause the transcription of new IFNmRNA species. In addition, the increase in amounts of functional IFN-α mRNA in RNA from treated cells was accompanied by a similar increase in physical amounts of IFN-α mRNA confirming that butyrate and BrdUrd do indeed cause increased synthesis of the mRNA rather than changes in its translational activity or the specific activity of the IFN it codes for.

A similar investigation of the IFN-β mRNA could not be performed since it did not code for active IFN-β . However, similar poly(A)$^+$RNA samples from butyrate-treated, BrdUrd-treated and untreated cells were electrophoresed through an agarose gel, transferred to nitrocellulose filter and hybridized with labelled IFN-β cDNA as in Fig. 17. Fig. 26

Fig.26. Blot-transfer hybridization analysis of IFN-β mRNA from induced, treated and untreated cells. 20ug of poly(A)$^+$RNA extracted at 7h after induction from (1) butyrate-treated, (2) untreated and (3) BrdUrd-treated cells was electrophoresed through an agarose-methylmercury gel and transferred to a nitrocellulose filter. The immobilised RNA was then hybridized with ^{32}P-labelled IFN-β cDNA.

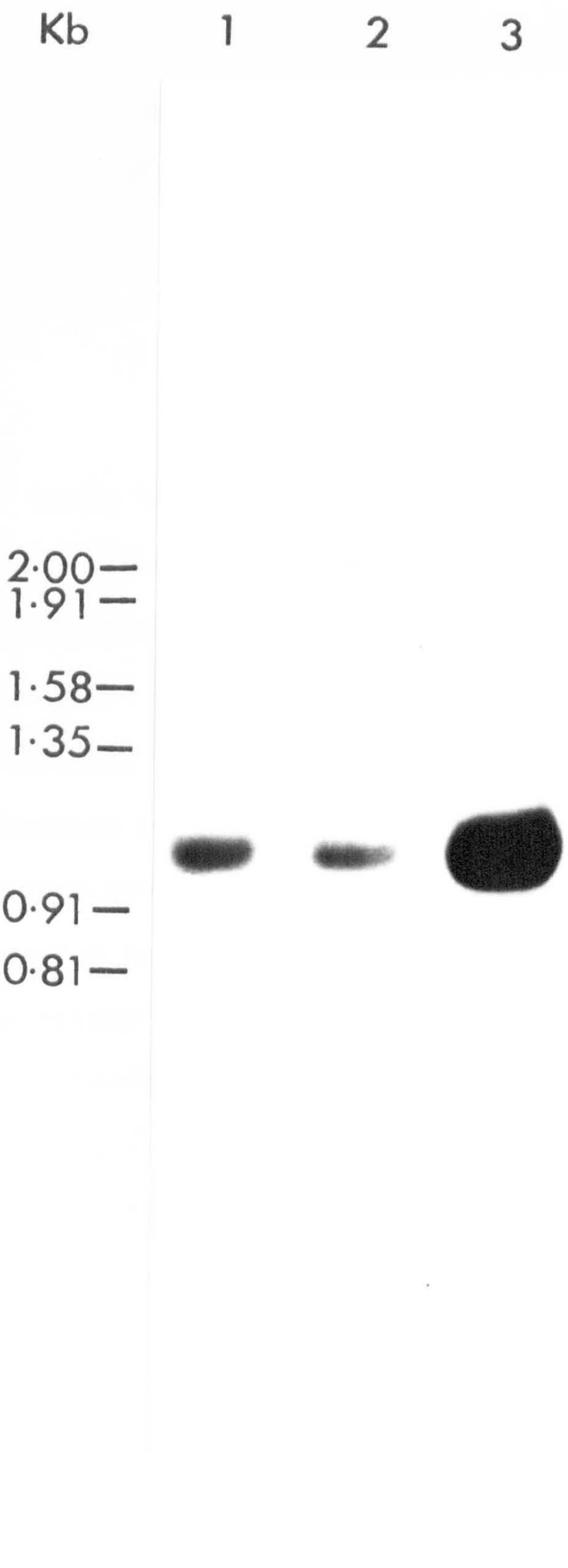


Fig. 26

shows that the IFN-β mRNA was the same size and also present in increased amounts in the RNA from treated cells. The increase in amounts of IFN-β , estimated from densitometric scans of the autoradiograph was 17-fold in RNA from butyrate-treated and 2-fold in RNA from BrdUrd-treated cells. These values were virtually identical to those for IFN-α mRNA shown in Fig. 18, therefore butyrate and BrdUrd treatments coordinately increased the synthesis of both IFN-α and IFN-β mRNA.

h) Discussion

These results have shown that Sendai virus induced the synthesis of IFN-α mRNA and also a non-functional IFN-β mRNA in the Namalwa cells used in these experiments. The size of the RNA hybridizing with IFN-α 2 cDNA probe ranged from 1.2 Kb to 1.35 Kb. Such size heterogeneity can be accounted for by inspection of cloned IFN-α cDNA and genomic DNA sequence data (Nagata et al., 1980; Goeddell et al., 1981). The minimum predicted size for IFN-α 1 and IFN-α 2, two of the species suggested to be expressed by Namalwa cells (see section 1.2), is approximately 1.08 Kb (based on a DNA sequence consisting of 70 bp of 5' leader sequence, 564-567 bp of coding region, 242 bp of 3' untranslated sequence and an estimated poly(A) tract 200 residues long). IFN-α 3 is also thought to be expressed by Namalwa cells since its predicted amino acid sequence closely matches the NH_2-terminal sequence of the major constituent of Namalwa IFN (Zoon et al., 1980). IFN-α 3 has a 342 bp 3' untranslated region therefore the predicted size of its mRNA in 1.18 Kb. Similarly, IFN-α 8mRNA, with a 441 bases of 3' untranslated region, would be even larger with a size of 1.28 Kb. In addition the 3' flanking regions of IFN-α genes have multiple possible sites for polyadenylation. When the IFN-α 1 gene is expressed after transfer

to mouse L-cells the majority of transcripts are polyadenylated at the first site, however two other sites are utilised resulting in a small proportion of transcripts which are 300 bases longer (S. Nagata, personal communication), that is up to 1.38 Kb in size. Admittedly, the identity of the IFN-α species expressed in Namalwa cells has not been confirmed however, these are possible explanations for the heterogeneity of IFN-α mRNA.

The non-functional IFN-β mRNA induced in these Namalwa cells, was discrete, only RNA 1.05 Kb in size hybridized with the IFN-β cDNA probe, which is the reported size of the single IFN-β mRNA . in fibroblasts (Raj and Pitha, 1981). SI mapping has shown that the IFN-β induced in Namalwa cells is indistinguishable from fibroblast IFN-β mRNA (Gross et al., 1982), therefore both are probably transcribed from the same unique gene. The IFN-β mRNA induced in the Namalwa cells used in the experiments described here, although not functional, comigrated with functional IFN-β mRNA from induced MG 63 cells (data not shown) suggesting that it was a full-length transcript. Therefore it was unlikely that its failure to code for active IFN-β was due to a large deletion or premature termination of transcription. The characteristics of this IFN-β mRNA are discussed further below.

The size distribution of IFN-α mRNAs in the fractionated poly(A)$^+$RNA from these cells was identical when determined either by oocyte translation or by hybridization with IFN-α cDNA probe. Therefore its heterogeneity represents functional mRNA and not incomplete or partially degraded transcripts. Furthermore the larger 1.9 Kb IFN-α and IFN-β transcripts reported by Sagar et al., 1981 were not detected by either method. Therefore the only IFNmRNA species produced by these Namalwa cells were of the size predicted from the IFN-α and IFN-β gene sequences.

The value obtained for the relative abundance of IFN-α mRNA agrees with information obtained from cDNA cloning and the ^{35}S-methionine labelling of IFN-α produced by these cells. IFN-α mRNA was estimated to represent 0.01% of the poly(A)$^+$RNA population in induced, untreated cells. By comparison only 2 IFN-α cDNA clones were identified in 2000 cDNA clones prepared from size fractionated poly(A)$^+$RNA from induced, butyrate-treated cells (Slocombe et al., 1982), suggesting a relative abundance of 0.1% for the IFN-α mRNA in this sample. Furthermore, the relative rate of synthesis of IFN-α protein was estimated to be 0.2-0.6% of total protein synthesis in induced butyrate-treated cells (see section 3.4). The difference between the values obtained from untreated and butyrate-treated cells is consistent with, and can be explained by the effect of butyrate on IFN and IFNmRNA synthesis. The following calculations were performed to test whether the estimated abundance of IFN-α mRNA could account for the IFN activity produced by the cells from which the RNA was extracted.

An average of 10pg of total RNA/cell was obtained during the course of these experiments. An average of 2% of this was recovered following oligo dT chromatography, giving a value of 0.2 pg of poly(A)$^+$ selected RNA/cell. If 0.01% of this poly(A)$^+$RNA was IFN-α mRNA, then there were 2×10^{-5} pg of IFN-α mRNA/cell.

The average molecular weight of IFN-α mRNA (Average size 1.27 Kb) was 4.19×10^5, therefore 4.19×10^{-17} pg of IFN-α mRNA were equivalent to 6×10^{23} molecules. This gives a value of $\frac{(2 \times 10^{-5})}{(4.19 \times 10^{17})} \times (6 \times 10^{23})$

or <u>28 molecules of IFN-α mRNA/cell</u>

The cells from which this RNA was extracted produced $10^{2.2}$ units of IFN/10^6 cells/h. Taking the specific activity of IFN-α to be

approximately 2 x 10^8 units/mg (Zoon et al., 1979) the cells produced $\frac{(10^{2.2})}{10^6 x\ (2x10^8)}$ = 5 x $10^{-12.8}$ mg of IFN-α/cell/h.

The molecular weight of IFN-α is 1.9 x 10^4 (see section 1.2), therefore 1.9 x 10^7 mg of IFN-α are equivalent to 6 x 10^{23} molecules. This gave a value of $\frac{(5 \times 10^{-12.8}) \times (6 \times 10^{23})}{1.9 \times 10^7}$ = $1.6 \times 10^{4.2}$ molecules of IFN-α/cell/h or 412 molecules of IFN-α/cell/min

If there are 28 molecules of IFN-αmRNA/cell, then 15 molecules of IFN-α are produced/IFN-α mRNA molecule/min, this was equivalent to 2475 amino acids/min (166 amino acids/molecule see section 1.2) Assuming the rate of elongation in eukaryotic cells ranges from 120-480 amino acids/min (Palmiter, 1975) this rate of protein synthesis would require 5-20 ribosomes to be associated with each IFN-α mRNA. Despite the number of assumptions and approximations made in this calculation, the value it gives for the size of the polysomes involved in IFN synthesis although rather high, is quite sensible. By measuring the IFN activity of nascent polypeptides associated with sucrose gradient fractionated polysomes, IFN was found to be associated with polysomes up to 8 ribosomes in size, (data not shown) therefore all of the available data are consistent with the conclusion that IFN-α mRNA is a low abundance mRNA representing approximately 0.01% of the poly(A)$^+$RNA synthesised by these cells. The importance of this, with regard to the limits it places on the study of IFNmRNA synthesis and degradation are discussed in section 3.1.

The IFN-β mRNA induced in these cells was defective since it did not translate to give active IFN-β, thus explaining the cells failure to produce detectable IFN-β activity. None of the characteristics which were investigated identified the reasons for

this lack of IFN-β mRNA function. Thus IFNmRNA of the predicted size, hybridizing specifically with IFN-β cDNA, was induced and regulated coordinately with IFN-α mRNA. This RNA was present in similar amounts, and specifically associated with polysomes to the same extent as functional IFN-α mRNA. The failure of this mRNA to translate active IFN-β could be explained by small deletions, insertions, substitutions or inversions in this IFN-β gene which would not necessarily affect expression of the gene or the regulation and function of the mRNA up to ribosome binding. Such mutations could cause faults in polypeptide synthesis or termination or the translation of an inactive protein. An unsuccessful attempt was made to identify the possible presence of an inactive translation product of this IFN-β mRNA using immunoprecipitation of ^{35}S-methionine-labelled cell and microinjected oocyte proteins with anti-IFN-β serum. To have been successful this would have required that the inactive IFN-β was still recognised by anti-serum to active IFN-β. However, insufficient radioactivity was incorporated into IFN-α proteins which served as a positive control, therefore no conclusions could be drawn.

One of the most significant conclusions of this section is that apart from the detection of inactive IFN-β, the results obtained by oocyte translation of IFNmRNA have been confirmed by hybridization with IFNcDNA. Hence the observations made in sections 3.1 and 3.2 relating to the control of IFNmRNA synthesis and degradation and the relative amounts of IFNmRNA in butyrate-treated, BrdUrd-treated and untreated cells have been substantiated. Therefore it can be stated that non-induced Namalwa cells do not contain detectable amounts of IFNmRNA. Infection with Sendai virus induces the coordinate synthesis and regulation of IFN-α and IFN-β mRNA. Treatment of the cells with butyrate or BrdUrd before IFN induction causes the synthesis of increased amounts of IFN-α and IFN-β mRNA which appear identical

in size distribution to the IFNmRNAs induced in untreated cells. This is consistent with, but does not prove that these treatments cause a coordinate increase in synthesis of all the IFN-α mRNA species and IFN-β mRNA, suggesting that butyrate and BrdUrd affect the regulation of all the IFNs expressed in these cells.

Section 3.4 A comparison of the proteins synthesised by butyrate - and BrdUrd-treated Namalwa cells

a) Introduction

Treatment of Namalwa cells with butyrate or BrdUrd has been shown to substantially increase IFN synthesis by up to 300-fold and 35-fold respectively (see section 3.2). Subsequent experiments have logically been directed towards characterising the effects of these treatments specifically on the control of IFNmRNA and IFN synthesis. However as an alternative line of investigation it would be useful to examine the effects of butyrate and BrdUrd on the expression of other proteins in Namalwa cells. This would establish firstly the extent to which these agents affect the rate of synthesis of other protein, and secondly, whether they induce any changes which are comparable to, or could account for the increased rate of IFN synthesis. This section compares the proteins synthesised by butyrate-treated, BrdUrd-treated and untreated Namalwa cells. Using polyacrylamide gel electrophoresis to resolve ^{35}S-methionine labelled proteins, the pattern of proteins synthesised and changes in their rates of synthesis have been assessed. The results of these experiments have now been published (Shuttleworth et al., 1982)

b) One-dimensional SDS-polyacrylamide gel analysis

In order to obtain information which could be related to the effects on IFN synthesis it was decided to investigate the proteins synthesised by Namalwa cells after 48h treatment with butyrate or BrdUrd, both before and 7h after induction by Sendai virus. Any changes which arise before or during the induction and synthesis of IFN could then be assessed.

Cells were treated for 48h with butyrate or BrdUrd then labelled

for 30 min with ^{35}S-methionine. The labelled proteins were compared by one-dimensional SDS-polyacrylamide gel electrophoresis using a 10-20% exponential gradient of polyacrylamide. From Fig. 27 it can be seen that the proteins synthesised by BrdUrd-treated and untreated cells were indistinguishable (Fig. 27, tracks 2 and 3). However, butyrate treatment affected the rate of incorporation of label into several proteins, which have been indicated by arrows (Fig. 27, track 1). The increase in one band migrating with an apparent molecular weight of 35,000 (indicated by large arrow), which for convenience will be referred to as butyrate enhanced protein BEP_{35}, was consistently seen, whereas a decrease in intensity of three bands in the molecular weight range of 12,000-18,000 (indicated by small arrows) occurred only occasionally.

Fig. 28 shows a similar analysis of protein from treated and untreated cells at 7h after induction of IFN synthesis. The preparation of Sendai virus normally used to induce IFN synthesis contains a high proportion of DI particles (see section 1.3) and will therefore be referred to as DI Sendai virus. Although DI Sendai virus is an efficient inducer of IFN, it does not replicate in Namalwa cells (Baker et al., 1980). Consequently the synthesis of virus polypeptides was almost undetectable (Fig. 28, tracks 1-3). Therefore a purified preparation of normal Sendai virus was used to infect cells in order to observe the effect of treatment on the synthesis of virus structural peptides. Synthesis of the L,P,HN,Fo and NP virus polypeptides and C, a virus-specific cellular protein (see section 1.3) were unaffected by the treatments (Fig. 28, tracks 4-6). Likewise the synthesis of an unidentified virus-specific cellular protein (indicated by dotted bar) was not affected. This protein has not been detected by others, but it was also induced in normal Sendai virus infected chick embryo fibroblasts and was

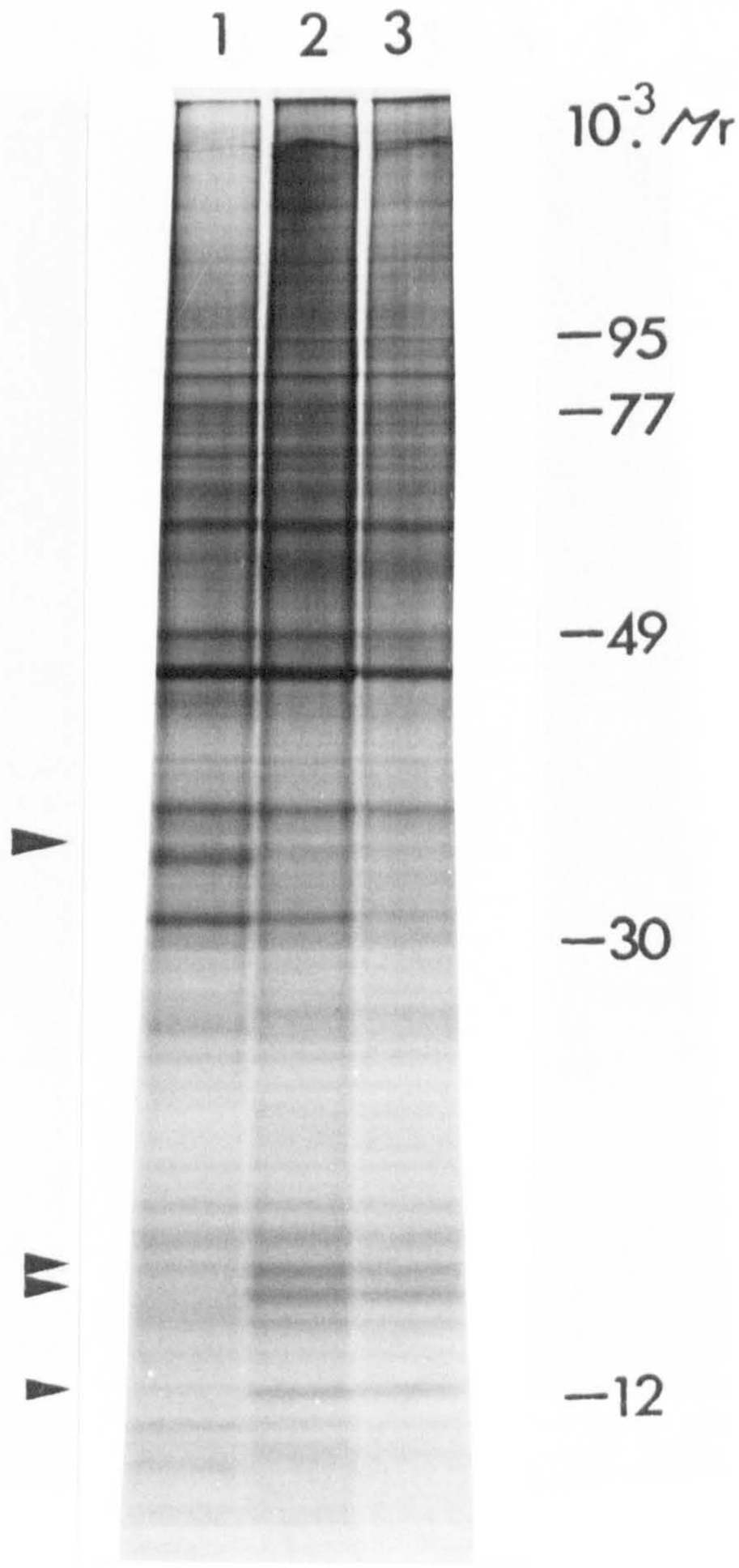


Fig.27. One-dimensional SDS-polyacrylamide gel analysis of proteins from treated and untreated cells. Cells were incubated for 48h in 0.8mM butyrate, 25ug/ml BrdUrd or maintainance medium then labelled with ^{35}S-methionine for 30min. Proteins from butyrate-treated (track 1), BrdUrd-treated (track 2) and untreated cells (track 3) were compared by electrophoresis through one-dimensional SDS-polyacrylamide gels. The large arrow indicates a reproducible change and the small arrows non-reproducible changes in the rates of synthesis of proteins from butyrate-treated cells.

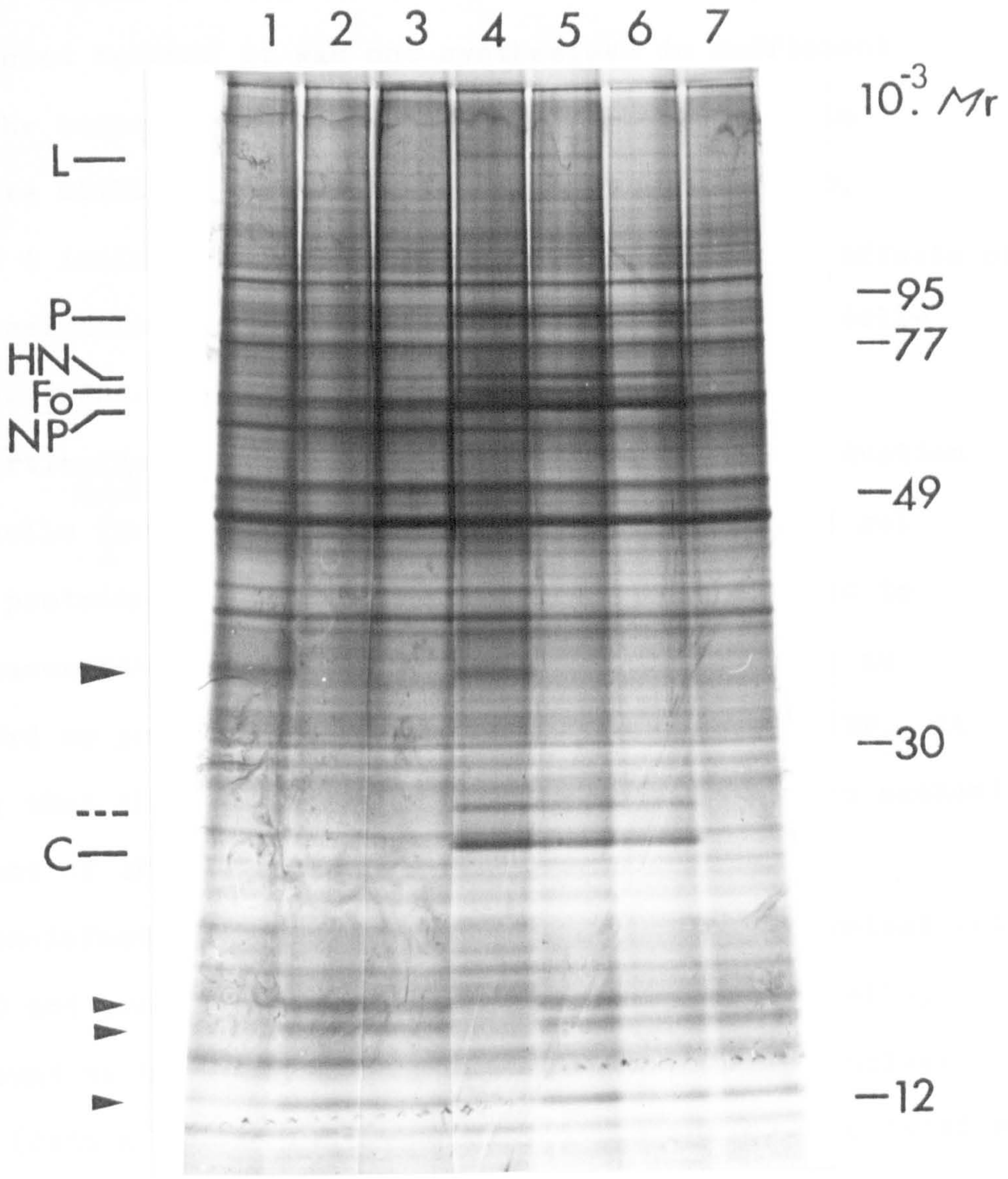


Fig.28. One-dimensional SDS-polyacrylamide gel analysis of proteins from Sendai virus-infected, treated and untreated cells.Cells were incubated for 48h in 0.8mM butyrate, 25ug/ml BrdUrd or maintainance medium then infected with either DI Sendai virus (tracks1-3) or normal Sendai virus (track 4-6). After 7h incubation the cells were labelled for 30min with ^{35}S-methionine. Cellular and virus protein synthesis was compared in butyrate-treated (tracks 1,4), BrdUrd-treated (tracks 2,5) and untreated cells (tracks 3,6). Track 7 shows untreated,uninfected control cells. The large arrow indicates a reproducible change and the small arrows indicate non-reproducible changes in the rates of synthesis of proteins from butyrate-treated cells. The letters refer to virus structural and virus-specific cellular proteins.

therefore not unique to Namalwa cells. The Sendai M polypeptide was not detected because it was not synthesised in sufficient quantity. The butyrate-induced changes in cellular protein synthesis were still detectable in infected cells (Fig. 28, tracks 1 and 4 indicated by arrows) and remained the only effects of treatment observable on one-dimensional gel separation of cell-associated proteins.

Glucocorticoids have been reported to enhance IFN production by Namalwa cells (Adolf and Swetly, 1979b). However one-dimensional gel analysis of proteins from prednisolone-treated cells failed to show any enhancement of BEP_{35} (data not shown). Therefore as neither BrdUrd or prednisolone affected the synthesis of BEP_{35} it was unlikely that this protein was associated with a common mechanism of enhancement of IFN synthesis.

When non-infected, butyrate-treated cells were homogenised using Nonidet P-40 and centrifuged to remove nuclei and intact cells, BEP_{35} was found to be located predominantly in the post nuclear supernatant (data not shown). Therefore BEP_{35} was not associated with chromatin, unlike some of the proteins reported to be induced or enhanced by butyrate in other cells (D'Anna et al., 1980).

The effects of treatment were characterised further by pulse-chase labelling of cells. Treated and untreated cells were labelled for 30min and followed by a 2h chase. The labelled proteins were separated by one-dimensional SDS polyacrylamide gels as in Figs. 27 and 28. Fig. 29 shows that apart from BEP_{35} no other changes in protein synthesis were apparent. By comparing the intensity of the band from pulse labelled (track 1) and pulse-chase labelled cells (track 2) it was clear that BEP_{35} was rapidly turned over, in fact after a 4h chase no enhancement was observed (data not shown).

The proteins secreted by treated and untreated cells were

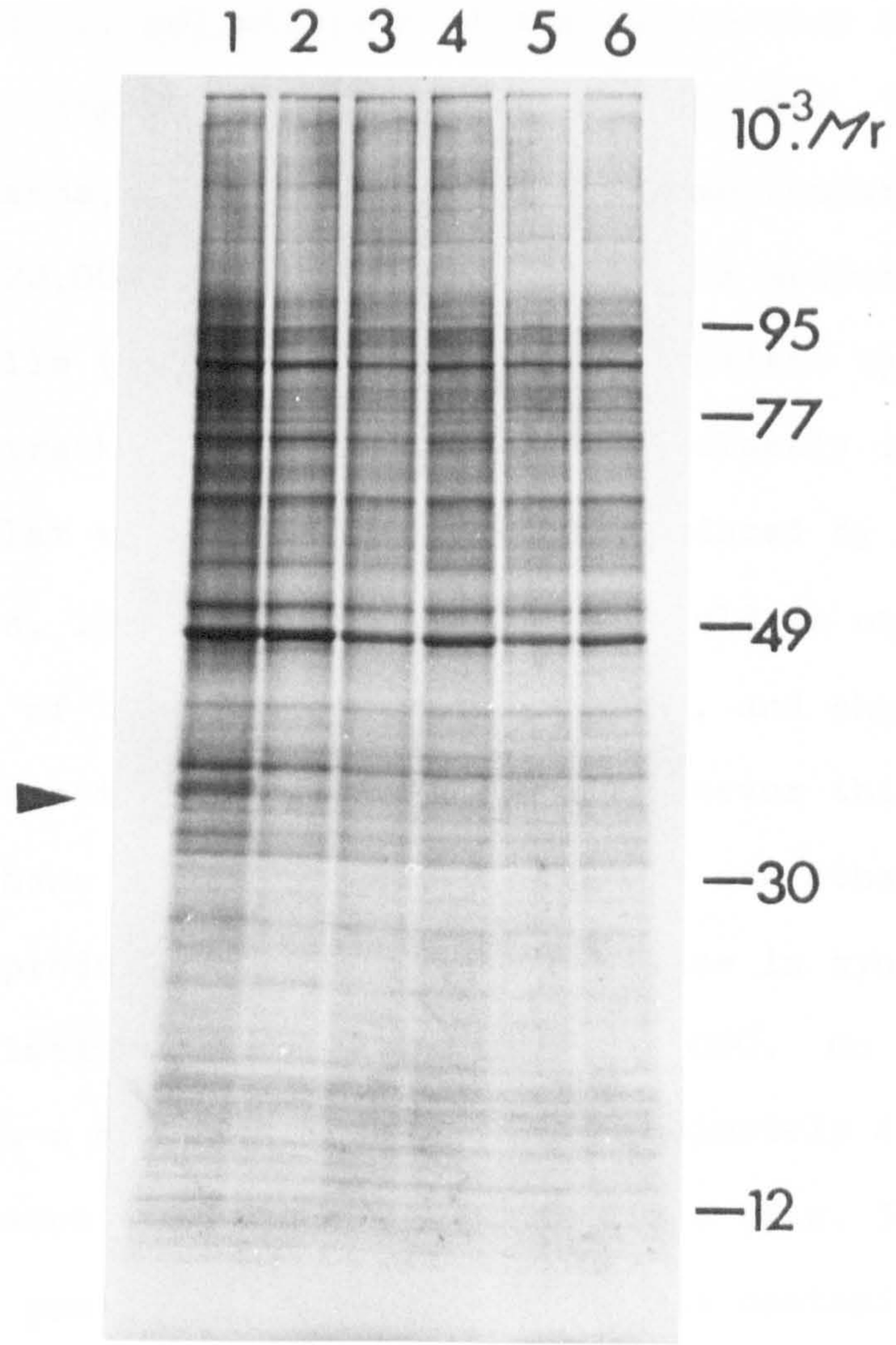


Fig.29. One-dimensional SDS-polyacrylamide gel analysis of pulse-labelled proteins from treated and untreated cells. Cells were incubated for 48h in 0.8mM butyrate, 25ug/ml BrdUrd or maintainance medium then either pulse-labelled for 30min with ^{35}S-methionine or pulse-labelled for30min with ^{35}S-mehtionine followed by a 2h chase with unlabelled methionine. Pulse-labelled (tracks 1,3,5) and pulse-chase labelled (tracks 2,4,6) samples from butyrate-treated (tracks 1,2), BrdUrd-treated (tracks 3,4) and untreated cells (track's 5,6) were compared by electrophoresis through one-dimensional SDS-polyacrylamide gels.

compared by one-dimensional gel analysis of the pulse-chase medium obtained from the labelling of cells used in Fig. 29. Fig. 30 shows that two broad bands, migrating with apparent molecular weights of 20,000 and 22,000 were enhanced in proteins secreted by butyrate-treated cells (track 1) and to a lesser extent by BrdUrd-treated cells (track 2). These corresponded closely in size to the reported molecular weights of IFN species produced by Namalwa cells (Allen and Fantes, 1980). Such an increase would be expected in view of the effects of treatment on IFN synthesis, and should be within the limits of detection (see discussion). However this by no means confirmed that these two bands were IFN. Only one other change was detected reproducibly, this was an increase in synthesis of a protein with a relative molecular weight of 11,000. On the gel shown, one protein with a molecular weight of approximately 45,000 appeared to show increased labelling in treated cells (Fig. 30 tracks 1 and 2). This protein most likely represents contamination of the sample by actin, a major cellular protein. No such change in labelling of this protein was observed in cell-associated samples (Figs. 27 and 28), therefore this suggested that the variation was caused by differences in contamination of the secreted proteins by cellular proteins.

c) <u>Two-dimensional gel electrophoresis</u>

One-dimensional gel electrophoresis is limited in its capacity to resolve individual proteins, therefore two-dimensional analyses were performed using either isoelectric focussing (IEF) or non-equilibrium PH gradient gel electrophoresis (NEPHGE) for the first dimension.. With the combination of ampholines used, the IEF gels were capable of resolving protein within a pI range of 4.5-6.3. More basic proteins would be separated by the NEPHGE gels.

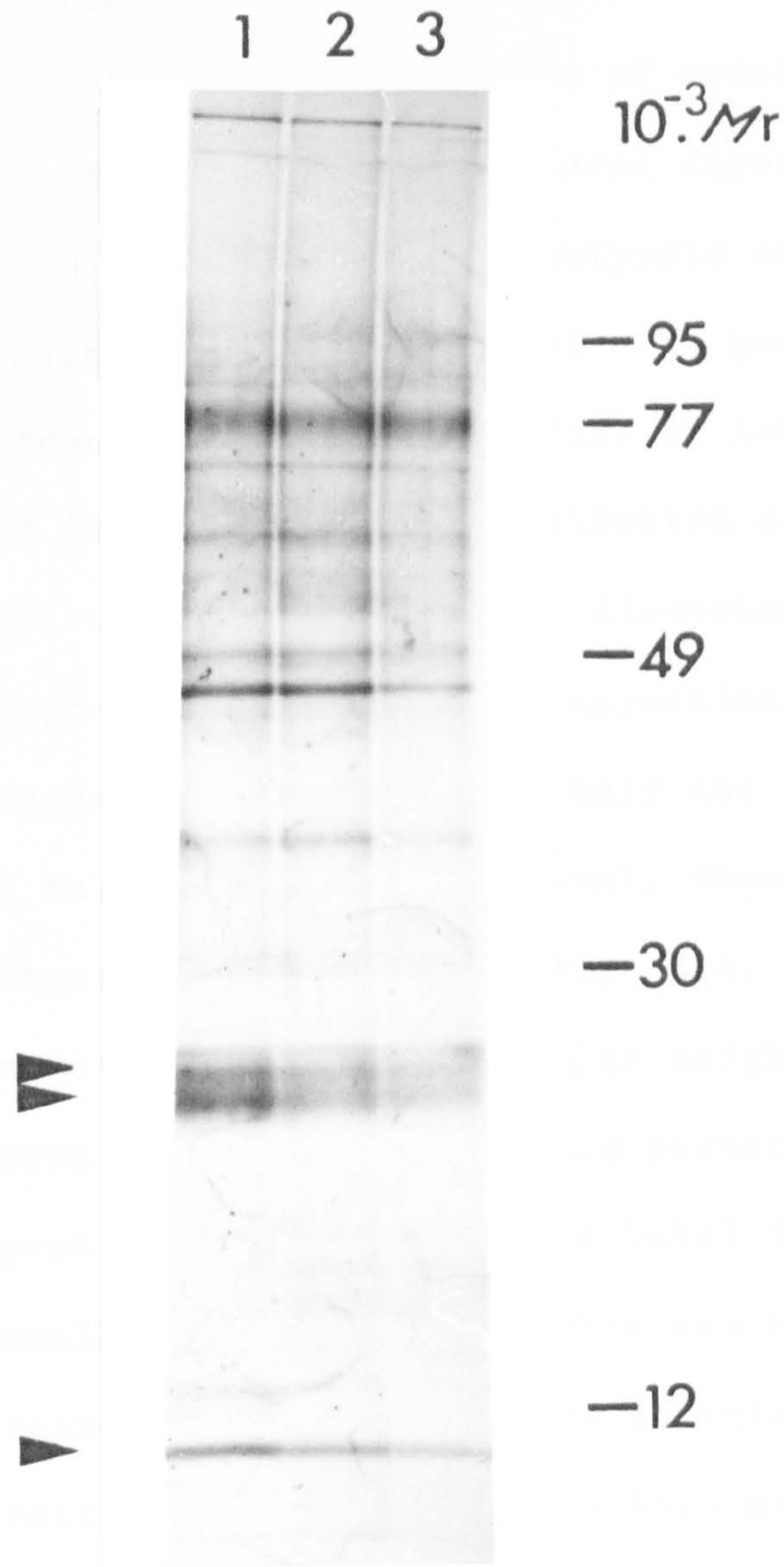


Fig.30. One-dimensional SDS-polyacrylamide gel analysis of secreted proteins from treated and untreated cells.The supernatants from the pulse-chase labelled cells in Fig.29 were used to compare the proteins secreted by butyrate-treated (track 1), BrdUrd-treated (track 2) and untreated cells (track 3). The arrows indicate proteins showing increased incorporation of label in treated cells.

Exponential gradient SDS-polyacrylamide gels were used for the second dimension because of their increased range of resolution and also to aid interpretation as they could be compared directly with the one-dimensional gels. Cells were treated with butyrate or BrdUrd and labelled with ^{35}S-methionine as above, except a 1h period of labelling was used to increase incorporation. Fig. 31 A-C shows proteins from butyrate-and BrdUrd-treated and untreated cells before induction, separated using a NEPHGE first dimension. With the autoradiograph exposure used (14 days) approximately 250 proteins could be detected by this system. Only two proteins were found to be affected reproducibly by treatment, these showed increased synthesis in butyrate-treated cells (Fig. 31A, indicated by large plus signs). One of these had a molecular weight of 35,000 and probably corresponds to BEP_{35}. On this particular gel an additional 8 polypeptides showed increased label incorporation (Fig. 31A, indicated by small plus signs), but this was not observed on other occasions. No reproducible changes were detected on gels of protein from BrdUrd-treated cells, although on this gel 12 polypeptides show reduced intensity (Fig. 31B, indicated by small minus signs).

The increase in incorporation of label into BEP_{35} caused by butyrate was quantitated by excising that section of the gel and comparing the radioactivity it contained with the same gel from untreated cells. The increase varied from 3-4 fold. As this was the most significant of the changes detected it showed that butyrate and BrdUrd treatment had relatively little effect on the synthesis of proteins other than IFN.

Aliquots of the samples separated on NEPHGE gels were separated using a IEF first dimension. Fig. 31, D-F shows that again very few changes were apparent. With a 14 day autoradiograph

Fig.31. Two-dimensional gel electrophoresis of proteins from treated and untreated cells. Cells were incubated for 48h in 0.8mM butyrate, 25ug/ml BrdUrd or maintainance medium then labelled for 60min with ^{35}S-methionine. Labelled proteins from butyrate-treated (A,D) , BrdUrd-treated (B,E) and untreated cells (C,F) were compared by electrophoresis through NEPHGE first-dimension (A-C) or an IEF first-dimension (D-F) followed by SDS-polyacrylamide gels for the second-dimension. Large plus signs indicate reproducible changes and small plus or minus signs indicate non-reproducible changes in the rates of synthesis of proteins.

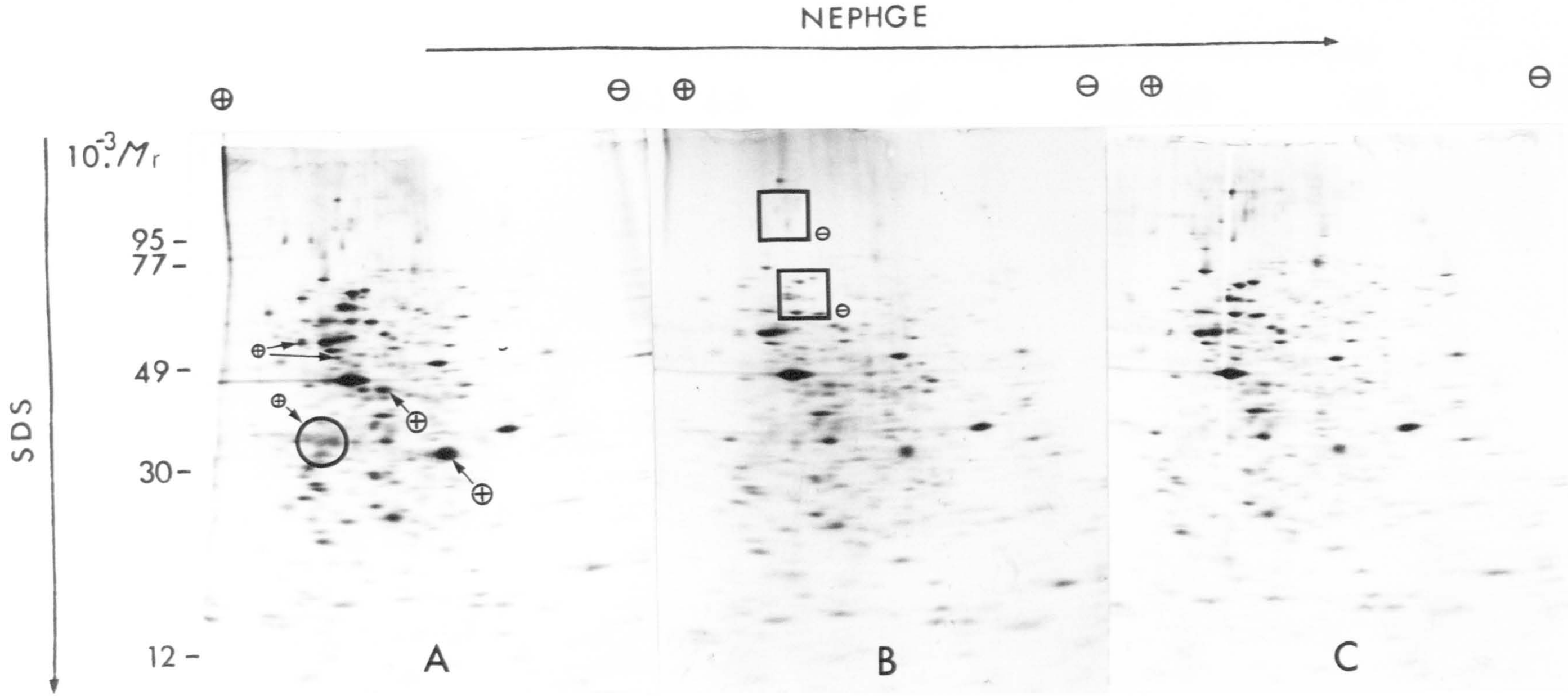


Fig.31

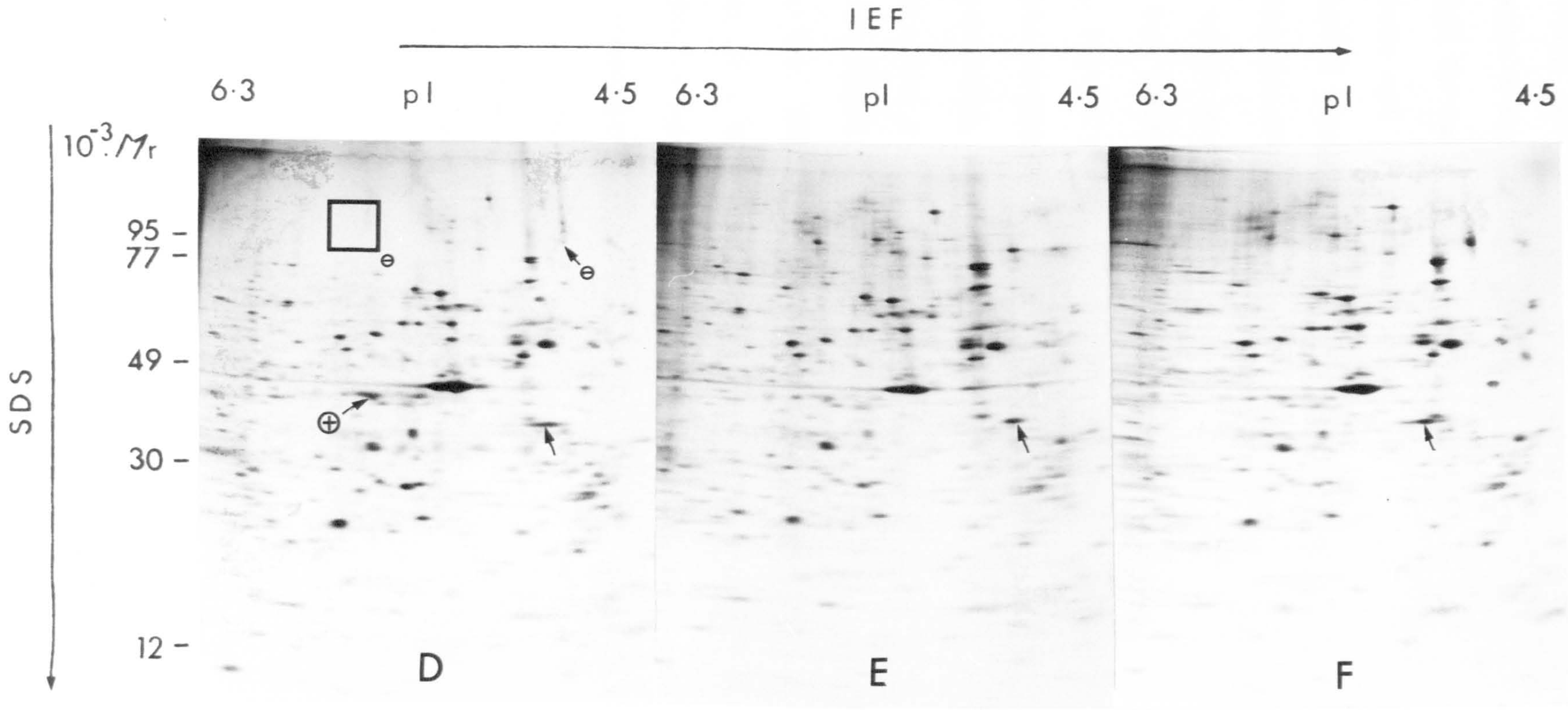


Fig. 31

exposure 300 proteins were detected using this system. Butyrate treatment consistently increased the incorporation of label into only one protein with a molecular weight of approximately 42,000 (Fig. 31D, indicated by large plus sign). On this gel a group of 4 other proteins were not visible and one protein showed reduced incorporation of label (indicated by small minus sign) but this was not observed reproducibly. The pattern of proteins synthesised by BrdUrd-treated cells was indistinguishable from untreated cells (Fig. 31, EandF) One protein showed differences in migration in the IEF dimension, its pI varying from 4.65-4.75, but this fluctuation was independant of treatment and may reflect modification during sample preparation. The 42,000 molecular weight proteins enhanced by butyrate and observed on the IEF and NEPHGE gels (Fig. 31A and D, indicated by large plus signs) are probably the same protein. However, BEP_{35} was not detected on the IEF gels, probably because it is a basic protein, since its position on NEPHGE gels suggests it has a pI greater than 7.0.

Fig. 32, A-C shows proteins from treated and untreated cells at 7h after induction using DI Sendai virus, separated by an IEF first dimension. Again butyrate consistently enhanced only one protein (Fig. 32A, indicated by large plus sign). On the gel shown a group of 4 other proteins showed increased synthesis (indicated by small plus signs). BrdUrd treatment resulted in no reproducible changes although on the gel shown one protein had increased synthesis (Fig. 32B, indicated by small plus sign). The position of Sendai virus structural polypeptides were determined by electrophoresing proteins from normal Sendai virus infected cells. Only the HN, P and Fo polypeptides were identified on the basis of molecular weight alone (Fig. 32E) and none of these were apparent in samples from DI Sendai virus infected cells.

Fig.32. Two-dimensional gel electrophoresis of proteins from treated and untreated cells following Sendai virus infection. Cells were incubated for 48h in 0.8mM butyrate, 25ug/ml BrdUrd or maintainance medium then infected with DI Sendai virus. 7h after infection the cells were labelled with ^{35}S-methionine for ,60min. Proteins from butyrate-treated (A), BrdUrd-treated (B) and untreated cells (C) were electrophoresed through an IEF first-dimension and SDS-polyacrylamide second-dimension. Labelled proteins from normal Sendai virus-infected,untreated cells (E) and uninfected, untreated cells (D) were prepared and separated in the same way. Large plus signs indicate reproducible changes and small plus and minus signs indicate non-reproduible changes in the rates of synthesis of proteins. The letters refer to virus structural proteins.

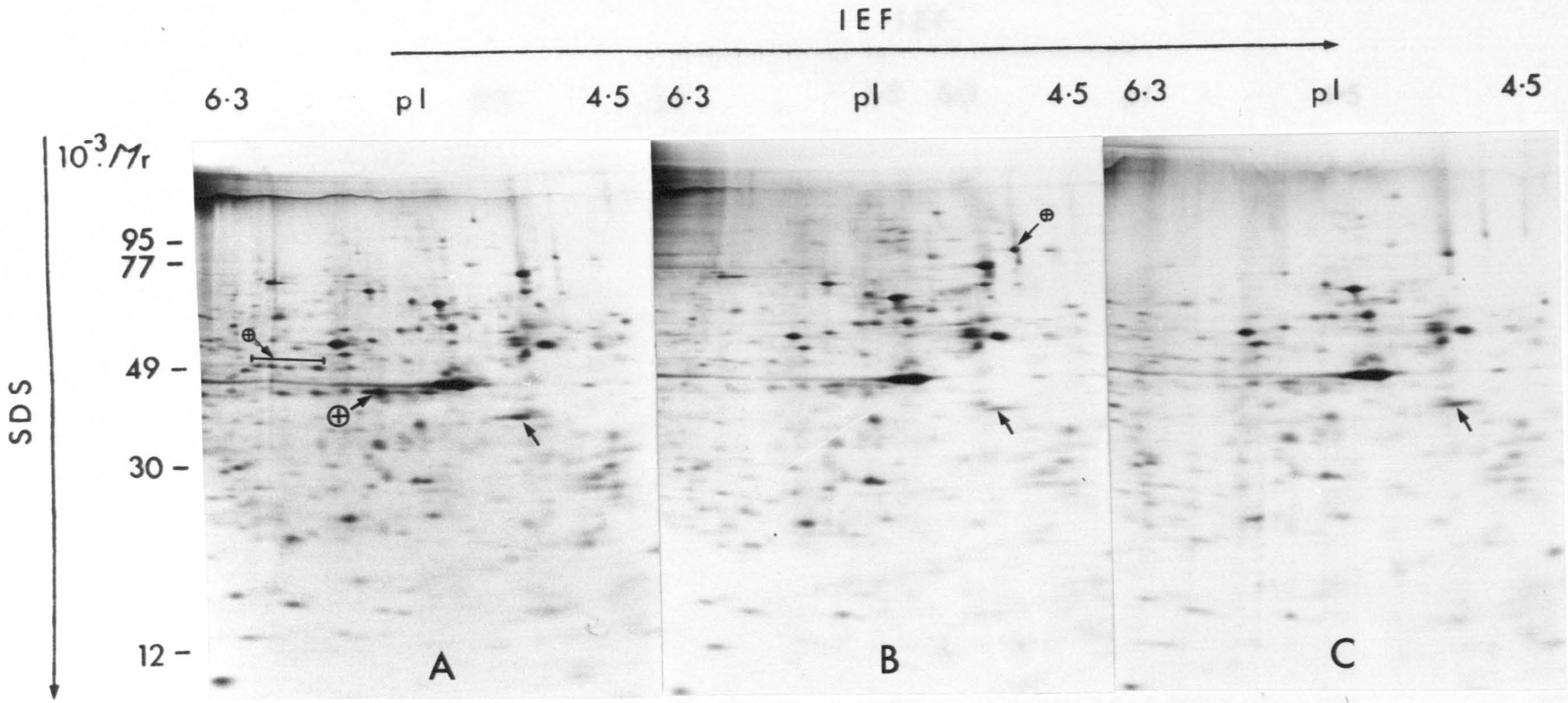


Fig. 32

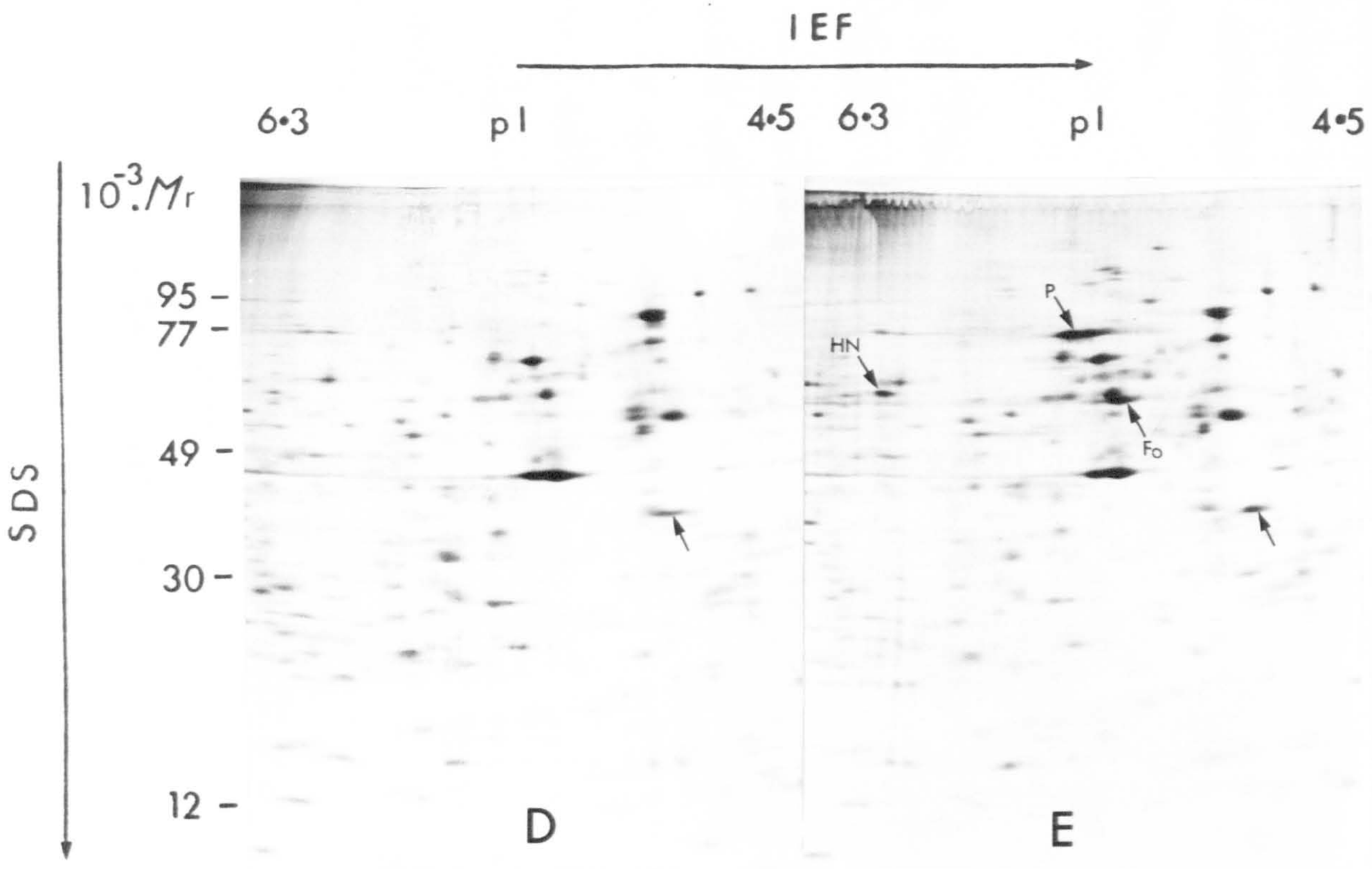


Fig. 32

Similar observations were made from analysis of samples from infected cells when resolved using NEPHGE first dimensions (data not shown), therefore it was concluded that butyrate and BrdUrd have a very limited effect on the synthesis of proteins in induced and non-induced Namalwa cells. At most only 12 proteins of the 300 investigated had altered rates of synthesis, of these only 2 proteins were reproducibly affected in butyrate-treated cells and none in BrdUrd-treated cells.

d) Discussion

This analysis of ^{35}S-methionine labelled proteins has shown that butyrate and BrdUrd treatment of Namalwa cells causes only minor changes in the relative rates of synthesis of only a few proteins when examined either before or after induction with Sendai virus. Most of these changes were not reproducible, only occurring on single occasions, and these probably reflect slight variations in the cells or artifacts arising from the running and processing of gels. Only two proteins were reproducibly affected by treatment, both showed increased incorporation of label following butyrate treatment. The most significant effect was the 3-4fold increase in BEP_{35} synthesis. Considering the magnitude of these effects they are clearly distinct from the reproducible increases in IFN synthesis caused by butyrate and BrdUrd.

Core histone acetylation induced by butyrate has been shown to give rise to chromatin with increased DN ase I sensitivity, a property characteristic of actively transcribed chromosomal loci (see section 1.1). As histone hyperacetylation has been demonstrated in butyrate-treated Namalwa cells (Baker et al. 1980) it is surprising that the consequences, in terms of expression of proteins,

are so slight. Likewise incorporation of BrdUrd into cellular DNA is also reported to alter DNA-protein interactions (see section 1.1) and might be expected to perturb the pattern of protein synthesis. Similar studies on butyrate-treated hepatoma cells also failed to detect large changes in protein synthesis (Rubenstein et al., 1979). In contrast considerable de novo changes in RNA and protein synthesis were reported in butyrate-induced erythroleukemic cells (Reeves and Cserjesi, 1979). IFN production by both erythroleukemic and Namalwa is similarly affected by both butyrate and DMSO (Swetly. 1976; Glazier et al., 1977). The nature of any response to butyrate treatment will obviously differ depending on a cell's phenotype and the genes it is expressing or capable of expressing.

Two-dimensional gel electrophoretic analysis using detection of proteins by staining or autoradiography is limited to relatively abundant proteins. Proteins detectable by their biological activity at normal cellular concentrations, such as IFN, are not always present in sufficient quantity to be detected by staining or autoradiography. It was estimated that a protein containing less than 0.002% of the radioactivity in a sample loaded onto IEF or NEPHGE gels would be below the limits of detection by the conventional autoradiography used here. This was determined by counting the radioactivity associated with one of the faintest spots on a typical autoradiograph and comparing this with the total amount of radioactivity recovered from the gel. By using the NK2 monoclonal antibody to IFN-α it was possible to estimate the proportion of radioactivity associated with IFN-α in pulse-chase supernatants. From 2-6% of the TCA-insoluble radioactivity in such supernatants from butyrate-treated cells was retained by an NK2-Sepharose column. It was therefore possible that labelled IFN could be detected by polyacrylamide gel analysis of secreted proteins as in

Fig. 30. Only 10% of the total TCA-insoluble radioactivity incorporated into cells during a 1h pulse was secreted, therefore IFN-α synthesis represents approximately 0.2-0.6% of total protein synthesis in butyrate-treated cells. Measurements of IFN activity showed that only 2% of the IFN synthesised by cells during a 1h period remained associated with the cells (see section 3.2) therefore as an approximation only 0.004-0.012% of the radioactivity incorporated into cell-associated proteins in the pulse-labelled samples used for two-dimensional gel analysis would be IFN. In this case it was unlikely that IFN could have been detected by the IEF or NEPHGE gel analysis used here, since the amount of radioactivity associated with each of the several IFN-α proteins would be below the limits of detection. Consequently, other low abundance proteins could be equally affected by treatment but not detected by this survey. Greater sensitivity of detection could have been obtained using fluorography or longer autoradiograph exposures, however very few additional proteins were detected when longer exposures were used. Unless butyrate or BrdUrd preferentially affect the expression of low abundance proteins, the 300 proteins detected here are a sufficiently large sample.

By infecting cells with normal Sendai virus it was clear that the treatments did not affect the synthesis of virus polypeptides which confirms the conclusions of Baker et al., 1980 that the increase in IFN synthesis is not caused by effects on the replication of the inducing virus.

In conclusion, while butyrate and BrdUrd have a marked affect on the synthesis of IFN, the synthesis of the majority of proteins expressed by Namalwa cells remains unaltered. No changes were detected which were comparable to, or could account for, the enhancement of IFN synthesis. These reasults suggest that whereas

butyrate and BrdUrd may have a general effect on the organisation and transcriptional capacity of chromatin, the pattern of proteins expressed is not necessarily disrupted.

SECTION 4. GENERAL DISCUSSION.

a) Post-transcriptional control of IFN synthesis?

The results presented in section 3 clearly show that IFNmRNA is inactivated and degraded during the shut-off of IFN production in Namalwa cells. It is most likely that this event, together with the cessation of IFN gene transcription, is responsible for the shut-off of IFN production. The inactivation of IFNmRNA in Namalwa cells can be delayed by incubating the cells at reduced incubation temperatures, and it is proposed that this effect is caused by inhibition of the synthesis of an RNA or protein which is involved in the degradation of IFNmRNA. By analogy with the effect of metabolic inhibiters on poly(rI).poly(rC) induced fibroblasts, the effect of reduced incubation temperature on IFNmRNA inactivation and IFN production in Namalwa cells is consistent with the post-transcriptional repressor model (see section 1.2). This proposes that the inactivation of IFNmRNA by a specific repressor molecule, induced at or shortly after the induction of IFN, is responsible for controlling IFN production. To date, the only evidence for such a repressor is the variation in IFNmRNA stability in cells perturbed by metabolic inhibiters.

It is conceivable that such a repressor protein is induced. Poly(rI).poly(rC) treatment of fibroblasts not only induces IFN synthesis but also a number of other mRNAs and proteins (Raj and Pitha, 1980a; 1980b). Similarly, it has been found that three regions of DNA, linked to but unrelated to the IFN-β gene, were coordinately induced by poly(rI).poly(rC) in fibroblasts (Gross et al.,1981).However it was found that the mRNAs derived from these regions were differentially expressed in poly(rI).poly(rC) induced fibroblasts and virus induced Namalwa cells. One of the mRNAs was not found in Namalwa cells, therefore it was unlikely to be involved in controlling IFNmRNA stability. At present

the function of these mRNAs and proteins remains unknown.

One possible candidate for the repressor molecule, a latent endonuclease which is activated as a consequence of the effect of IFN itself, does not appear to be involved in controlling IFNmRNA stability. IFN increases the synthesis of an enzyme called 2'-5' oligoadenylate synthetase which when activated b y double-stranded RNA (such as viral nucleic acid) produces the oligonucleotide 2'-5' oligoadenylate (reviewed by Lengyel,1981). The only known function of 2'-5' oligoadenylate is to activate the latent endonuc - RNase L. Thus it would be possible for IFN secreted by induced cells to activate the RNase L and cause the degradation of IFNmRNA. However, thestability of IFNmRNA was found to be unaffected by varying levels of 2'-5' oligoadenylate synthetase, and further more the level of enzyme remained unchanged during normal induction of IFN synthesis in fibroblasts (Sehgal and Gupta, 1980). Therefore RNase L is unlikely to be involved.

There are alternative expla nations for the inactivation of IFNmRNA other than those involving a <u>specific</u> post-transcriptional <u>control</u> mechanism. There is very little evidence to indicate that the inactivation of IFNmRNA is specific (see section 1.2). IFNmRNA could belong to a class of mRNAs which are rapidly turned over by constitutive mechanisms. Hence the observation that the inactivation of IFNmRNA is not accompanied b y changes in the stability of total ^{3}H-uridine labelled mRNA (Sehgal and Gupta,1980) would not be remarkable.Consequently the idea that IFNmRNA is controlled is open to question. Indeed there is no evidence to suggest that IFNmRNA stability changes during the period of IFN production, as would be required if IFN synthesis was controlled post-transcriptionally. The effects of metabolic inhibiters and low temperature on IFNmRNA stability could equally be explained by the inhibition of a constitutive

process (with short half-life components) which is involved in determining the stability of a class of mRNAs to which IFNmRNA belongs. The rapid decline in IFNmRNA would then be explained solely by the control of transcription.

In order to confirm how IFN production is controlled, following the initial events of induction, measurements of the rate of transcription of the IFN genes, and the rate of degradation of IFNmRNA, are required. Preliminary attempts to determine the rate of transcription in induced Namalwa cells (see section 3.1) by pulse-labelling intact cells with ^{3}H-uridine were unsuccessful since insufficient radioactivity was incorporated into IFN gene transcripts. However it should be possible to determine the rate of IFN gene transcription by incorporation of ^{32}P-labelled nucleotide triphosphates into "run-off" transcripts in isolated nuclei and hybridization with cloned IFNcDNA (Derman et al., 1981). The rate of IFNmRNA degradation could likewise be determined by following the decay of RNA hybridizing with IFNcDNA during a chase of ^{3}H-uridine labelled cells. These measurements would indicate at what level (s) IFN gene expression is controlled.

An alternative approach to the question of post-transcriptional control would be to look for changes in IFNmRNA stability by following the decay of functional IFNmRNA in the presence of inhibiters of RNA synthesis. In a preliminary experiment in which cordycepin was added to Namalwa cells at 8h after induction, the levels of IFNmRNA in the cells during the following 2h (measured by translation in oocytes) declined with a half-life of approximately 30mins (data not shown). This was significantly shorter than the normal half-life of 2.7h (see section 3.2) indicating that transcription was still occuring after 8h while IFNmRNA levels were declining. By comparing th e stability of IFNmRNA at various times after induction it should be possible to determine whether its stability is controlled during

the shut-off of IFN synthesis. The obvious criticism of this approach would be that inhibition of RNAsynthesis would interfere with other non-specific regulatory processes such as those suggested above. Nonetheless any change in IFNmRNA stability would be informative since it would most likely be a consequence of IFN induction.

To conclude, although the data in section 3.1 are consistent with the post-transcriptional repressor hypothesis, they do not prove that IFNmRNA stablity is controlled during the shut-off of IFN production in Namalwa cells.

b) <u>IFN gene expression in Sendai induced Namalwa cells</u>.

IFN-α and IFN-β gene expression are shown to be coordinately induced and regulated in Sendai virus induced Namalwa cells (see section 3.3). This observation is not unexpected, since both classes of IFN are induced by the same inducer. However, as the IFN-α and IFN-β genes are differentially expressed in different cell-types and with different inducers, it was conceivable that the two classes of IFN gene were independantly controlled and hence could have shown different kinetics of induction and regulation.

The conclusion that the IFN-β mRNA expressed by the Namalwa cells used in these experiments doesnot code for an active IFN-β protein is perhaps more suprising (see section 3.3). Other Namalwa cells produce readily detectable amounts of IFN-β mRNA and protein (see sections 1.2 and 2). It was suggested that the IFN-β gene in the Namalwa cells used in these experiments has mutated, and work is in progress to characterise the gene from which the IFN-β mRNA is derived, using cloned genomic DNA from these cells.

Karyotype and serotype analysis have confirmed that the cells used in these experiments are Namalwa cells. Therefore the mutated

IFN gene must have arisen recently during culture. Whatever the reason , the failure to express active IFN-β in these cells has possible implications for the function of multiple IFN genes. Functional constraints are known to have an important role in regulating the rate at which mutated genes (or their products) become established in a population. This would suggest that a mutation of the IFN-β gene in cultured cells could only persist in the population if it was not essential in culture. In the intact organism, the rate of fixation of such a mutation must be much slower, as evidenced by the close homology of thr IFN-α and IFN-β genes, and the fact that a large number of these genes appear to code for active proteins. This might suggest that each of the functional IFN proteins has a distinct role <u>in vivo</u>.

The question of how differential expression of the IFN-α and IFN-β genes is controlled remains. Since expression of both classes of IFN gene is induced and regulated coordinately, the mechanism determining the relative proportions of each IFN produced must involve differential regulation at the transcriptional or post-transcriptional level (or possibly the translational level). Unfortunately no comparison of the relative proportions of IFN-α and IFN-β mRNA and proteins could be made in these Namalwa cells because no active IFN-β was produced. The relative proportions of IFN-α and IFN-β mRNA in these cells were however suprising. On a molar basis IFNmRNA was found to be composed of 29% IFN-α and 71% IFN-β . Since the composition of Namalwa IFN (in cells producing active IFN-β) was reported to be approximately 85% IFN-α and 15% IFN-β these IFNmRNA values would appear to be inconsistent. However it is possible that the differential regulation is affected in the Namalwa cells which are not producing active IFN-β.

To conclude, a systematic survey, comparing the relative proportions Of IFN-α and IFN-β mRNA and protein is required in order to determine the level at which differential expression of the IFN genes occurs. Ideally measurements of IFN protein, using specific antisera or monoclonal antibodies should be made, in conjunction with hybridization of IFNmRNA with cloned IFNcDNA probes in order to elliminate possible differences in specific activity of the proteins or translational activity ofthe mRNA. The investigation could be extended by comparing the expression of the multiple IFN-α genes, using probes specific for each IFN-α gene. Such probes could be constructed from the 3' untranslated regions of the genes where least homology occurs. This information would hopefully enable the design of further experiment, possibly using <u>in vivo</u> or <u>in vitro</u> transcription systems to investigate transcriptional control of cloned IFN genes.

c) <u>The effect of butyrate and BrdUrd on IFN gene expression</u>.

Treatment of Namalwa cells with butyrate or BrdUrd before induction by Sendai virus has been shown to increase the synthesis of both IFN-α and IFN-β mRNA (see sections 3.2 and 3.3). The increase in IFN-α mRNA levels in treated cells partly explains the increase in IFN production which has previously been reported (see section 1.3). An increase in the efficiency of translation of IFNmRNA also contributes to the increased IFN yield, but this was apparently caused by culture conditions prior to induction of cells.

No qualitative differences could be detected in either the IFNmRNA or IFN expressed by treated cells. It is most likely that an increase in expression of a single IFN-α species would be detected either by changes in the size distribution of IFN-α mRNA or biological properties of the IFN produced. However these data do not prove

conclusively that treatment coordinately increases the synthesis of all IFN-∝ species expressed by Namalwa cells.

Further analysis of the effects of butyrate and BrdUrd on IFN gene expression will require measurements of the rate of transcription of the IFN genes in order to determine the level at which they act. Butyrate and BrdUrd could well prove useful in studying the mechanisms which control IFN gene transcription (or mRNA processing) since their effect must be mediated by alterations in these events. However at present too little is known about the mode of action of butyrate and BrdUrd to propose how such experiments could be designed.

d) Conclusions.

Sendai virus induced Namalwa cells have provided useful information about the control of IFN-∝ and IFN-β gene expression in cultured humancells. Although key questions still remain unanswered the information provides a basis for further experiments on the control of IFN synthesis. Of the remaining problems, the nature of the proximate inducer and the mechanisms controlling transcription of the IFN genes are probably the most important. However even when these control processes are described, this information will have to be related to the in vivo situation in order to understand the physiological significance and function of this group of genes. Clearly there is a considerable distance to go before the IFN system is fully understood.

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