

# Identification of individual interferon-producing cells by *in situ* hybridization

(murine  $\alpha$ -interferon/murine  $\beta$ -interferon/Newcastle disease virus)

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**ABSTRACT** Individual interferon (IFN)-producing cells were identified by hybridization *in situ* followed by autoradiography. cDNAs corresponding to murine IFN- $\alpha$  and murine IFN- $\beta$  labeled by nick-translation to high specific activity ( $2\text{--}4 \times 10^8$  dpm/ $\mu\text{g}$ ) with  $\alpha$ - $^{35}\text{S}$ -labeled dATP were used as probes for hybridization with IFN mRNA in mouse C-243 cells induced with Newcastle disease virus. Control experiments with non-induced cells or with non-IFN-related labeled DNA monitored the specificity of the autoradiographic signal. Under optimal conditions of IFN induction, between 15% and 40% of the cells gave a hybridization signal with a mixture of IFN- $\alpha$  and - $\beta$  probes. Differential hybridization with either the IFN- $\alpha$  or - $\beta$  probe or a mixture of both, at three different time intervals after induction, revealed that only a small fraction of cells had detectable amounts of IFN- $\alpha$  mRNA, whereas in the majority of the positive cells IFN- $\beta$  mRNA was present.

Studies on the genetics and on the cellular origin of interferon (IFN) production have suffered from the limitation that the results represented average values obtained from cultures or from cell populations *in vivo*, because there was no method to identify and characterize individual IFN-producing cells at the same time. To be able to study IFN production at the single cell level is of obvious importance, because it can provide answers to several questions—for example, the number of IFN-producing cells in a given culture system or the nature and the number of IFN-producing cells *in vivo* as a function of the inducing agent. In the present study, we have used cDNA probes of murine IFN- $\alpha$  and - $\beta$  to develop a method for detecting the presence of specific mRNA by *in situ* hybridization. This method makes it possible to distinguish individual IFN-producing cells that can, at the same time, be stained for morphological identification.

## MATERIALS AND METHODS

**Labeling of cDNA Probes.** The recombinant plasmids pMIF 1204 (carrying a partial cDNA for murine IFN- $\alpha$ 2) and pM  $\beta$ -3 (carrying the complete nucleotide sequence for murine IFN- $\beta$ ) have been described (1, 2). After digestion with *Pst* I, the plasmids were electrophoresed through 1% agarose slab gels and the inserts [820 base pairs (bp) for IFN- $\alpha$  and 680 bp for IFN- $\beta$ ] were recovered from the gel slices by electroelution and chromatography on DE-52 columns. The inserts were labeled to high specific activity ( $2\text{--}4 \times 10^8$  dpm/ $\mu\text{g}$ ) with  $\alpha$ - $^{35}\text{S}$ -labeled dATP ( $>1000$  Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) in a nick-translation reaction essentially as described by Haase *et al.* (3). Briefly, a 25- $\mu\text{l}$  reaction mixture contained 400 ng of DNA template/50 mM Tris-HCl, pH 7.4/10 mM MgCl<sub>2</sub>/1 mM dithiothreitol/50  $\mu\text{g}$  of nuclease-free bovine serum albumin per ml (Bethesda Research Laboratories)/30  $\mu\text{M}$  of each dCTP, dGTP, and

dTTP/250  $\mu\text{Ci}$  of  $\alpha$ - $^{35}\text{S}$ -labeled dATP/5 units of DNA polymerase I/100  $\mu\text{g}$  of DNase I. The mixture was incubated at 15°C for 2 or 4 hr and incorporation of labeled nucleotides was determined by trichloroacetic acid precipitation in the presence of carrier DNA. Because we found previously that 30%–40% of the labeled nucleotide incorporated after 2 hr with no further increase at 4 hr, we routinely stopped the nick-translation reactions after 2 hr. For control experiments, phage QX174 DNA digested by *Hae* III (Bethesda Research Laboratories) was labeled as described above. The major fragment sizes of the nick-translated cDNA probes ranged from 100 to 200 bp as determined by electrophoresis through an 8% polyacrylamide gel under denaturing conditions.

**Induction of IFN.** C-243 cells were seeded in 75-ml Falcon flasks at a density of  $1.5 \times 10^6$  cells per flask in minimal essential medium supplemented with 10% newborn calf serum. Twenty-four hours later, sodium butyrate was added to 1 mM, and the cells were incubated for another 48 hr before induction (4, 5). Medium was then removed and the cell monolayer was infected with Newcastle disease virus (NDV) (Kumarov strain) at an input multiplicity of 15–20 plaque-forming units (pfu) per cell. In preliminary experiments, we found that maximal and identical IFN production occurred at input multiplicities ranging from 3 to 20 pfu per cell. Further increasing the multiplicity resulted in decreased IFN production. Two hours later, the virus was removed, and the cells were washed with phosphate-buffered saline, and were incubated with prewarmed medium. For determination of cumulative IFN production, aliquots of medium were removed from the flasks at different time intervals. Supernatants were acidified to pH 2 to inactivate residual virus for at least 48 hr before titration. IFN assays were performed on mouse L cells as described (6).

**Preparation of Cells for *in Situ* Hybridization.** After removal of medium, cells were washed with phosphate-buffered saline, detached from the plastic by vigorous shaking of the culture flask, and dispersed by repeated pipetting. They were then centrifuged for 10 min at  $300 \times g$ , resuspended in phosphate-buffered saline supplemented with 2% newborn calf serum, and adjusted to  $2 \times 10^5$  cells per ml. Approximately 20,000 cells (100  $\mu\text{l}$ ) were deposited on a coated glass slide (3) by spinning in a cyto centrifuge (Shandon) at 500 rpm for 5 min. After air drying, the preparations were fixed for 20 min with ethanol/acetic acid (3:1). To increase diffusion of the probe through the cell membrane, the preparations were treated with 0.2 M HCl for 20 min, briefly washed in water, incubated at 70°C in  $2 \times \text{NaCl/Cit}$  ( $1 \times \text{NaCl/Cit} = 0.15$  M NaCl/0.015 M Na citrate), washed again, and digested for 15 min at 37°C with 1  $\mu\text{g}$  of proteinase K per ml (Boehringer Mannheim) in 20 mM Tris-HCl, pH 7.4/2 mM CaCl<sub>2</sub> (preliminary experiments comparing concentrations from 0.5 to 5  $\mu\text{g}/\text{ml}$  of proteinase K had shown 1  $\mu\text{g}/\text{ml}$  to be optimal).

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Abbreviations: NDV, Newcastle disease virus; IFN, interferon; pfu, plaque-forming units; bp, base pair(s).

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After a brief washing in water, the slides were dehydrated through 70% and 95% ethanol. For certain control experiments, cells were treated for 30 min at 37°C with a mixture of RNase A and RNase T1 (Boehringer Mannheim) as described (3).

To evaluate possible loss of RNA from the cells during these treatments, control experiments were performed as follows: sodium butyrate-treated C-243 cells were labeled with [<sup>3</sup>H]uridine (Amersham; 30 Ci/mmol) at a concentration of 2 μCi per ml of culture medium 2 hr after infection with NDV. Five hours later, cells were harvested and washed 5 times with phosphate-buffered saline supplemented with 2% newborn calf serum to remove excess [<sup>3</sup>H]uridine. As described above, cells were then centrifuged onto coated glass slides, fixed, and subjected to the treatments to increase diffusion of the probe into the cell. After autoradiographic exposure for 24 hr, grains were counted randomly over 20–40 cells per slide.

**Hybridization.** Hybridization was based on the method of Haase *et al.* (3). Unless stated otherwise, the cell preparations were hybridized with 3 ng of probe ( $\approx 1 \times 10^6$  dpm) in 10 μl of a solution consisting of deionized 50% formamide/0.6 M NaCl/10 mM Tris·HCl, pH 7.4/1 mM EDTA/sonicated salmon sperm DNA (250 μg/ml)/yeast tRNA (1 mg/ml)/poly(A) (100 μg/ml)/1× Denhardt's solution (7). After heating to 95°C for 2 min and chilling on ice, dithiothreitol was added to 10 mM. The solution was placed on the cells and covered with a siliconized glass coverslip, the edges of which were sealed with rubber cement. Hybridization was carried out in the dark at 22°C for 72 hr. Coverslips were then removed, the slides were rinsed in a wash medium consisting of 50% formamide/10 mM Tris·HCl, pH 7.4/1 mM EDTA/0.6 M NaCl, and transferred for 1 hr to 2× NaCl/Cit at 55°C. Finally, the slides were washed for 3 days (with stirring) in the wash medium and dehydrated through 70% and 95% ethanol containing 0.3 M ammonium acetate. For autoradiography, the slides were dipped into Kodak NTB-2 nuclear track emulsion that had been melted at 45°C and diluted 1:1 with 0.6 M ammonium acetate. The slides were then dried in an upright position and stored in a light-proof box at 4°C for 10–15 days. The slides were developed in Kodak D-19b developer at 15°C for 3 min, rinsed under running tap water for 1 hr, and stained with Giemsa's stain diluted 1:20 in 10 mM phosphate buffer.

For microscopic evaluation, the grains over a minimum of 200 cells were counted. A cell was scored as positive if the number of grains was at least 1.5× background level.

## RESULTS

**Kinetic Analysis of IFN mRNA Synthesis by *in Situ* Hybridization.** IFN was induced in sodium butyrate-treated C-243 cells with NDV at a multiplicity of 20 pfu per cell, and cumulative IFN production was determined (Fig. 1). In parallel, 2, 5, and 8 hr after induction, cells were deposited on slides by cytocentrifugation and were hybridized with a mixture of IFN-α and -β cDNA (3 ng of each probe per slide). Non-induced cells hybridized under identical conditions served as controls. The results of the hybridization are represented by the bars in Fig. 1, giving the percentages of positive cells (i.e., those with at least 1.5× the number of grains found over non-induced cells). As expected, the number of cells giving a positive signal increases between 2 and 5 hr after induction, followed by a slight decrease at 8 hr.

**Specificity of the Hybridization Signal.** The specificity of hybridization was ascertained by the following criteria: (i) hybridization of induced and non-induced cells with the same amount of an unrelated labeled control DNA (*Hae* III-digested phage QX174 DNA); (ii) hybridization of non-induced cells with the IFN-α and -β probes; (iii) treatment of

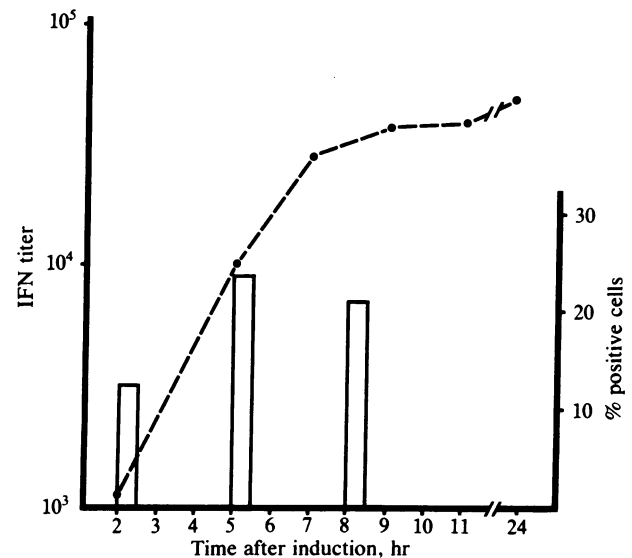


FIG. 1. Kinetics of IFN production and detection of mRNA in C-243 cells. ●—●, Cumulative IFN titers in supernatants from butyrate-treated NDV-induced C-243 cells on a log<sub>10</sub> scale. Bars represent percentage of cells harboring RNA sequences hybridizing to a mixture of IFN-α and -β cDNA (3 ng of each) 2, 5, and 8 hr after induction.

induced and non-induced cells with RNase prior to hybridization with the specific probes. The results of these controls are summarized in Table 1. Hybridization of either induced or non-induced cells with control DNA resulted in <10 grains per cell after 13 days' exposure. After hybridization with IFN-α and -β cDNA probes, ≈10% of the non-induced cells occasionally had up to 25 grains, but no difference was observed between sodium butyrate-treated and untreated control cells. This may indicate the presence of low amounts of IFN mRNA in a small number of non-induced cells, because after treatment with RNase none of these cells had more than 10 grains. Occasionally, in NDV-induced cells treated with RNase and hybridized with the IFN-α and -β probes, we observed that ≈10% of the cells had >50 grains; however, without RNase treatment, ≈10% of the cells had >200 grains (Fig. 2). When we hybridized induced cells with 10 ng of probe per slide, there was no increase in grain counts per cell (data not shown), indicating that hybridization with 3 ng of cDNA was already conducted under sufficient excess of probe.

As a further control, we examined whether treatment of the cell preparations to increase diffusion of the cDNA

Table 1. Specificity of hybridization signal

Treatment of C-243 cells				
Sodium butyrate (1 mM)	NDV	RNase	Hybridization mixture	No. of grains per cell
—	—	—	Control DNA*	<10
—	—	—	IFN-α and -β cDNA	<10–20
+	—	—	IFN-α and -β cDNA	<10–20
+	—	+	IFN-α and -β cDNA	<10
+	+	—	Control DNA	<10
+	+	+	IFN-α and -β cDNA	<10–50
+	+	—	IFN-α and -β cDNA	<10 to >200

Cells treated as indicated were hybridized with a mixture of 3 ng of each of the two IFN cDNA or with 5 ng of control DNA and were processed as described in *Materials and Methods*. Autoradiographic exposure was for 13 days.

\*Control DNA was phage QX174 DNA digested by *Hae* III and labeled to the same specific activity as the IFN cDNA probes.

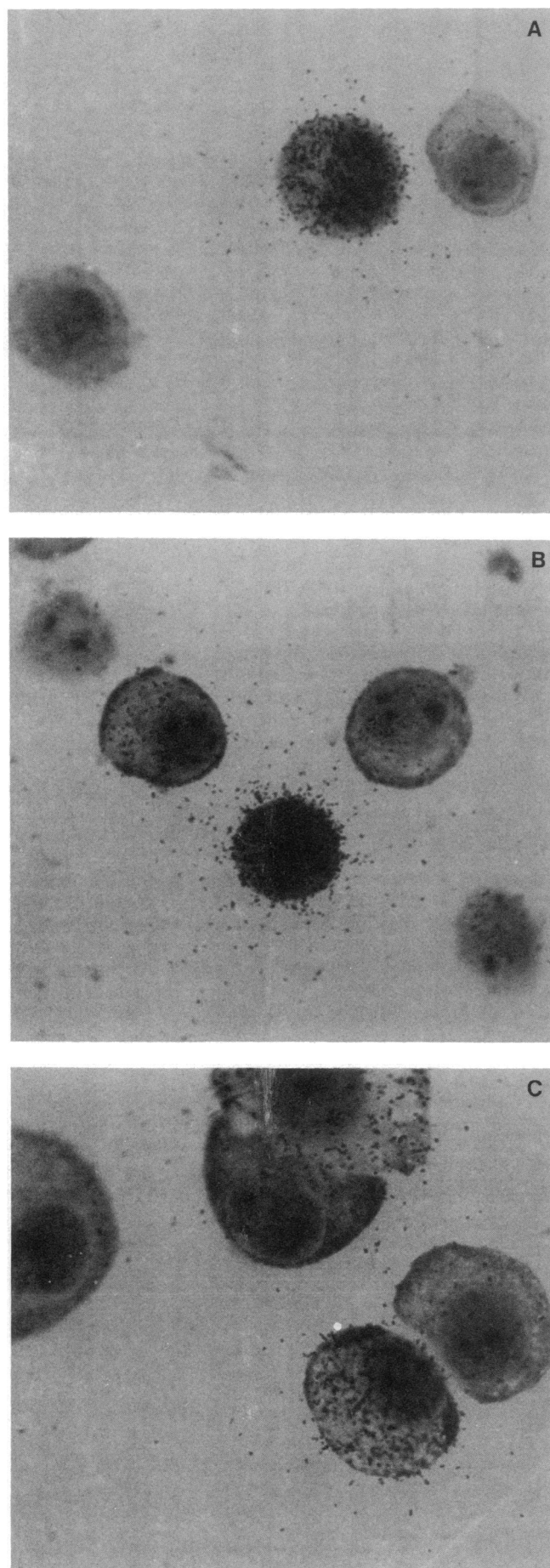


Table 2. Autoradiographic demonstration that no RNA is lost by C-243 processed for hybridization

	Slide no.	No. of cells counted per slide	No. of grains per cell ( $\pm$ SD)
Untreated cells	1	32	42.6 ( $\pm$ 17)
	2	32	39.2 ( $\pm$ 13)
Treated cells	1	37	43.3 ( $\pm$ 12)
	2	20	35.2 ( $\pm$ 17)
	3	27	40.8 ( $\pm$ 11)
Treated cells/ RNase	1	100	<3

Slide preparations of butyrate-treated C-243 cells induced with NDV and labeled with [ $^3$ H]uridine (2  $\mu$ Ci per ml of culture medium) for 5 hr were treated with 0.2 M HCl, 2 $\times$  NaCl/Cit at 70°C, and proteinase K (3  $\mu$ g/ml). Autoradiographic exposure was for 24 hr.

probe into the cells resulted in loss of RNA. Butyrate-treated C-243 cells, induced with NDV and labeled with [ $^3$ H]uridine (see *Materials and Methods*), were deposited on coated glass slides and fixed. Several slides were treated with 0.2 M HCl, 2 $\times$  NaCl/Cit at 70°C, and proteinase K at 3  $\mu$ g/ml, or with RNase as described above. All slides were then autoradiographed for 24 hr. As shown in Table 2, no loss of RNA occurred in cells processed for hybridization when compared to untreated cells, because we found identical distribution of silver grains over cells from both preparations. After RNase treatment, however, autoradiographic signals decreased to background levels.

**Differential Analysis of IFN- $\alpha$  and - $\beta$  mRNA Induction.** Induced cells were hybridized with 3 ng of either IFN- $\alpha$  or IFN- $\beta$  probe or a mixture of both (3 ng of each). The results are summarized in Fig. 3. It is evident that IFN- $\beta$  represents the major IFN mRNA species, present in 23% of the cells 5 hr after induction, whereas IFN- $\alpha$  mRNA was detected in only 7% of the induced cells at the same time. When a mixture of IFN- $\alpha$  and - $\beta$  probes was used, 30% of the cells were found to be positive. As we observed a great heterogeneity in grain count among positive cells, we differentiated between (i) weakly positive cells with 30–50 grains per cell, (ii) positive cells with 50–100 grains per cell, and (iii) strongly positive cells with >100 grains. With increasing time after induction, the total number of positive cells increases and the strongly positive cells appear (Fig. 3).

Table 3 summarizes the results obtained from different slide preparations to give an indication of the variability found within and between experiments. Each percentage given was obtained from a different slide.

**IFN Production by Cloned C-243 Cells.** Since in all hybridization experiments only some cells gave a positive signal with the IFN probes, it was important to investigate the possibility that only this fraction was capable of producing IFN. To this end, progeny of cloned individual cells were examined for IFN-producing capacity. A suspension of C-243 cells was diluted out into microtiter plates (Falcon plastics; 96 wells per plate) in 2-fold steps until a final dilution corresponding to 0.2 cells per well. The wells were then examined microscopically, and those containing a single cell were scored and subsequently followed up. Of the 56 wells scored

FIG. 2. *In situ* hybridization of NDV-induced mouse C-243 cells, 5 hr after onset of induction. (A) A mixture of  $^{35}$ S-labeled IFN- $\alpha$  and - $\beta$  probes (3 ng of each) was used. A positive and two negative cells can be seen. (B) Same mixture of probes as in A; a strongly positive cell, together with a slightly positive cell (upper left) and a cell with a number of grains that is just above background (upper right). (C) Hybridization with IFN- $\beta$  probe only; a positive cell can be seen next to a negative cell. Autoradiographic exposure was for 13 days. ( $\times$ 900.)

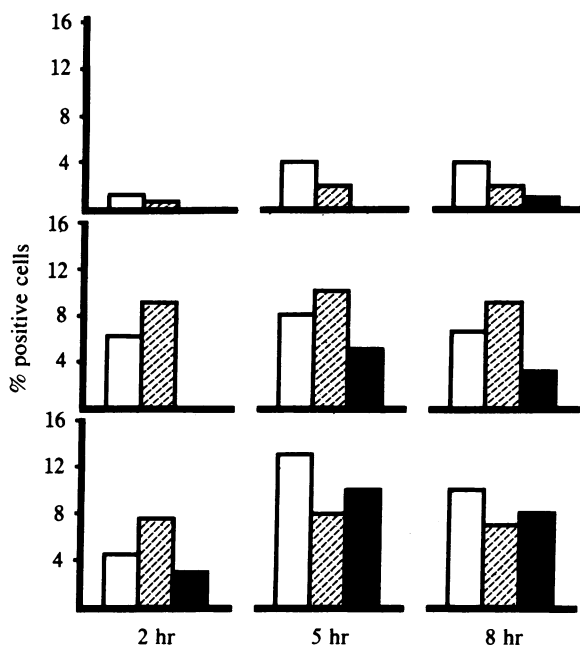


FIG. 3. Time course of appearance of detectable IFN mRNA in IFN-producing C-243 cells. Two, 5, and 8 hr after IFN induction, cells were hybridized with cDNA probes for either IFN- $\alpha$  cDNA (Top), IFN- $\beta$  (Middle), or a mixture of both (Bottom). Three nanograms of probe was used per slide, and autoradiographic exposure was for 13 days. Open bars represent percentage of cells with 30–50 grains; hatched bars represent cells with 50–100 grains; filled bars represent cells with >100 grains.

for the presence of a single cell, 30 gave rise to a proliferating clone. The cells from each clone were subcultured and induced with NDV. Although there was some variation in IFN titers, the 30 clones were all capable of IFN production. This showed that the C-243 cells used for the hybridization experiments did not consist of a mixed population of producer and non-producer cells.

## DISCUSSION

In the present study, we describe a method for detecting IFN mRNA by means of hybridization *in situ*. *In situ* hybridization has been described by Gall and Pardue (8) for detecting

Table 3. Percentage of positive cells after hybridization with either the IFN- $\alpha$  or IFN- $\beta$  probe or a mixture of both

Time after induction, hr	IFN cDNA probe*		
	$\alpha$	$\beta$	$\alpha$ and $\beta$
Exp. 1			
8	ND	24	40
8	ND	22	29
8	ND	14	15
8	ND	24	24
Exp. 2			
2	2	15	15
2	1	9	13
5	6	23	30
5	8	17	24
8	7	18	25
8	5	22	21

ND, not done.

\*Hybridization was done with 3 ng of either IFN- $\alpha$  or IFN- $\beta$  cDNA or a mixture of both (3 ng of each). Autoradiographic exposure was for 13 or 18 days.

amplified ribosomal gene sequences, and the method has been refined by Brahic and Haase (9) to permit detection of single copies of viral nucleic acids. The main problem in detecting IFN mRNA is the fact that the protein is usually only synthesized in low amounts. For example, for C-243 cells treated with sodium butyrate and induced with NDV, we calculated a production rate of  $\approx 0.01$  unit per cell per hr, corresponding to  $5 \times 10^{-3}$  pg, assuming that identical amounts of IFN are produced per cell (an assumption that the present work has shown to be incorrect). As a consequence, even in high producer cells such as C-243, IFN mRNA represents only a small fraction of the total cellular mRNA. Furthermore, IFN mRNAs are of relatively small size, varying from 1 to 1.5 kilobases (1, 10). Therefore, the theoretical specific activity per molecule of labeled cDNA probe is rather low, rendering difficult detection by autoradiography. In our approach, we have used  $^{35}\text{S}$ -labeled triphosphate deoxynucleotides to prepare by nick-translation radioactive cDNA probes with high specific activity ( $>2-4 \times 10^8$  dpm/ $\mu\text{g}$ ). This is 8–10 times more than specific activities usually obtained with  $^3\text{H}$ -labeled nucleotides. Furthermore, efficiency of grain development is higher for  $^{35}\text{S}$  (0.5 grain per disintegration) than for  $^3\text{H}$  (0.1 grain per disintegration) (11), and microscopic localization of grains is satisfactory.

In a kinetic study, we observed a good correlation between IFN titers in culture supernatants and hybridizing IFN mRNA in the cells. The specificity of the autoradiographic signal was ascertained by the very low grain count observed in uninduced cells treated with the IFN probes and in induced cells treated with a non-IFN-related DNA of the same fragment size and specific activity as the specific probes. Grain counts over non-induced cells were often higher after hybridization with the IFN probes than after hybridization with the control DNA (Table 1). This may indicate the presence of low levels of IFN mRNA in some uninduced C-243 cells, because the number of grains in these cells decreased after treatment with RNase. We have repeatedly observed a very low level of constitutive IFN synthesis in our line of C-243 cells (12). This could explain the presence of some cells with a faint positive signal even when uninduced. RNase treatment did not always completely abolish the hybridization signal in induced cells, although the number of grains in such cells decreased by at least 75%, as compared to induced cells without RNase treatment. We do not know the reason for this residual activity after RNase treatment; it may be due to the fact that a fraction of IFN mRNA has been reported to be relatively RNase resistant (13). In induced cells with a positive signal, we often observed an even distribution of grains over the whole cell. One interpretation of this could be the presence of hybridizable mRNA in the nucleus, although we feel that a more likely explanation is the trapping of cytoplasm over and under the nucleus during slide preparation.

The number of grains per cell in induced C-243 cells was very heterogeneous; some cells had >200 grains (Figs. 2 and 3), others had between 50 and 100 grains, and the majority of cells had only background levels, ranging from 10 to 20 grains per cell. This heterogeneity was quite pronounced at the three intervals after induction tested, with the appearance of strongly positive cells 5 hr after induction. This observation is indicative of a high degree of variability of IFN production in individual cells. It may be argued that during induction not every cell had been infected with NDV, even at the high multiplicity of infection used in our experiments; however, we were using the optimal amount of virus, as it is not possible to further increase IFN production by augmenting the input multiplicity. The cloning experiment has shown that all C-243 cells are intrinsically capable of producing IFN. Moreover, since the hybridization treatment does not result in a significant loss of RNA from the cells, as shown

by the control experiment with [<sup>3</sup>H]uridine-labeled cells, we can conclude that when C-243 cells are induced with NDV, only a fraction of the cells make IFN at any given time. The explanation for this is not clear at present, but we believe that this is suggestive of an influence of cell cycle on inducibility. Experiments in synchronized cells may be able to settle this question. It may also suggest that our method is as yet not sensitive enough to detect very low amounts of IFN mRNA.

The prevalence of cells (15%–23% depending on time after induction) giving a positive signal with the IFN- $\beta$  probe as compared to the results with the IFN- $\alpha$  probe is completely in line with previous work, showing that  $\approx$ 80% of IFN produced by NDV-induced C-243 cells migrates in NaDodSO<sub>4</sub>/polyacrylamide gels with an apparent molecular weight of 35,000, which corresponds to Mu IFN- $\beta$  (14, 15). Only from 1% to 7% of the induced cells gave a positive signal with the IFN- $\alpha$  probe, which is in line with the relatively low proportion of Mu IFN- $\alpha$  ( $\approx$ 20%) in NDV-induced C-243 IFN.

The low number of cells with hybridizable IFN- $\alpha$  mRNA makes it difficult at present to determine whether IFN- $\alpha$  and - $\beta$  are produced by the same or by different cells. As can be seen in Table 2, the number of cells positive for IFN- $\beta$  is sometimes equal to the number positive for IFN- $\alpha$  and - $\beta$  together, although in general a clear increase is observed when the two probes are used together, which would suggest that different cells make IFN- $\alpha$  and - $\beta$ . On the other hand, the number of strongly positive cells increases when both probes are used together, suggesting that the same cell synthesizes both IFN- $\alpha$  and - $\beta$  (Fig. 3). Evidently, we have not resolved this particular problem in the present experiments; a possible approach could be to hybridize the same preparation first with one probe, and after autoradiographic exposure, rehybridize with the second probe.

The lower percentage of positive cells with the IFN- $\alpha$  probe alone either indicates that some cells only make IFN- $\beta$  but not - $\alpha$ , or it may reflect the possibility that our IFN- $\alpha$  probe is only specific for a fraction of the IFN- $\alpha$  mRNAs induced. Currently, two different subtypes of murine IFN- $\alpha$  have been described: IFN- $\alpha_1$  and - $\alpha_2$ , and our probe corresponds to  $\alpha_2$  (1, 16). There is  $\approx$ 90% homology between the sequence of IFN- $\alpha_1$  and - $\alpha_2$ . Thus, our cDNA probe will readily detect IFN- $\alpha_1$  and - $\alpha_2$  mRNA but will fail to detect putative IFN- $\alpha$  mRNA sequences exhibiting only a low degree of homology to IFN- $\alpha_1$  or - $\alpha_2$ . This is, however, not very likely, because strong homology has been conserved during evolution between all members of the IFN- $\alpha$  gene family, even across the man–mouse species barrier (17).

The interpretation of *in situ* hybridization results is limited by the fact that this method detects mRNA and not the protein itself. Although, in general, a good correlation exists between the amount of mRNA synthesized and the IFN secreted, there has been one report demonstrating the presence of

untranslated human IFN- $\beta$  mRNA in induced Namalwa cells belonging to a particular clone (18). This, however, is not necessarily a disadvantage of *in situ* hybridization, because this technique could also help to identify cells in which IFN mRNA is transcribed but not translated.

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