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Interferon messenger RNA is produced constitutively in the organs of normal individuals

(gene expression/cytokines/S1 mapping)

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ABSTRACT **The use of RNA blot hybridization with DNA or RNA probes of high specific activity has shown that interferon (IFN)-α mRNA is present constitutively in the spleen, kidney, liver, and peripheral blood leukocytes of normal individuals. A single band ($\approx$1.2 kilobases) was detected in poly(A)$^+$ RNA isolated from human organs. This RNA hybridized specifically to human IFN-α_1 DNA and comigrated with mature IFN-α mRNA from virus-induced human peripheral blood leukocytes. No IFN-β RNA transcripts were detected in any of the tissues tested. IFN-γ mRNA was detected in only one sample of normal human spleen, which also contained an unusually high level of IFN-α mRNA. The use of a modified S1 mapping technique revealed the presence of IFN-α_1 and -α_2 transcripts only. No IFN-α_4, -α_5, -α_6, -α_7, -α_8, or -α_{14} transcripts were detected in the same sample. The detection, in all the samples tested, of a characteristic pattern of expression of IFN genes, different from that obtained following induction, together with the low number of transcripts present ($\leq$0.03 copy per cell) suggest that specific IFN genes are transcribed constitutively *in vivo*.**

Interferons (IFNs) constitute an important group of regulatory cytokines that modulate the expression of a number of cellular genes (1) and exhibit pleiotropic biological activities both *in vitro* and *in vivo* (2). In view of the biological importance of IFNs, it is of interest to determine how the expression of IFN genes is regulated *in vivo* under normal physiological conditions. Such information may also provide insight into more general aspects of gene control in eukaryotes.

The production of IFNs is usually considered to be subject to stringent control, and significant quantities of IFNs are produced only after induction (3–5). Thus, although human cells contain 18 or more gene loci encoding potentially functional IFNs (6, 7), the expression of these genes is usually not detected. Induction leads to the activation of one or more IFN genes, and transient transcription of the corresponding mRNA culminates in the synthesis of IFN protein (4, 5, 8). Recent evidence suggests, however, that IFNs may be produced *in vivo* under conditions other than virus infection (9, 10) and that these endogenous IFNs may play a role in regulating such important physiological processes as fetal development and normal hematopoiesis. As biologically active interferons are detected only irregularly and then in small amounts in the organs of experimental animals or humans in the absence of induction (10–13), we developed techniques based on a modified S1 mapping procedure to detect low levels of specific IFN RNA transcripts. These techniques permitted the systematic study of the expression of IFN genes in normal individuals. We show herein that transcription of certain IFN genes, but not others, could be detected in the organs of normal individuals in the apparent absence of induction. The characteristic pattern of expression of IFN genes *in vivo* together with the low number of IFN transcripts produced per cell suggest that specific IFNs are produced constitutively under physiological conditions.

MATERIALS AND METHODS

RNA Extraction. The organs of accident victims (whose organs had been taken for transplantation) were cut into small pieces and frozen immediately in liquid nitrogen. Buffy coats from 400 ml of blood taken on citrate from normal donors were treated twice with 9 vol of 0.98% NH_4Cl at 4°C to lyse erythrocytes. The leukocytes were then washed twice with phosphate-buffered saline at 4°C and frozen immediately in liquid nitrogen. Frozen organs were homogenized in lysis buffer [6 M urea/3 M LiCl/0.1% $NaDodSO_4$/heparin (20 units/ml)/10 mM sodium acetate, pH 5.0], and the RNA was precipitated at 0°C, extracted twice with phenol/chloroform, and chromatographed twice on oligo(dT)-cellulose. Low levels (<1.0 μg) of poly(A)$^+$ mRNA were recovered quantitatively by precipitating the RNA with 2.5 vol of ethanol at −20°C overnight in siliconized tubes and ultracentrifuging the RNA precipitates (100,000 × *g*; 1 hr).

RNA Blot Hybridization. RNA was electrophoresed in 1.5% agarose gels in the presence of formaldehyde (14), transferred to nylon filters (Hybond-N, Amersham) in the presence of 20× SSC [1× SSC (standard saline citrate) is 0.15 M NaCl/7.5 mM sodium citrate], and irradiated for 4 min at 312 nm (180 W). The RNA blots were prehybridized in 5× SSC/1× Denhardt's reagent (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/100 mM sodium phosphate buffer, pH 6.5/0.1% $NaDodSO_4$/denatured salmon sperm DNA (100 μg/ml) at 68°C for 4 hr. The RNA was then hybridized to either cloned human IFN genes {labeled by nick-translation to a specific radioactivity of $\geq 2 \times 10^9$ cpm per μg of DNA using [α-^{32}P]dATP and [α-^{32}P]dCTP (7000 Ci/mmol; Amersham; 1 Ci = 37 GBq)} or to Riboprobes {of similar specific activity synthetized *in vitro* using SP6 RNA-polymerase (15), [α-^{32}P]CTP, and [α-^{32}P]UTP (800 Ci/mmol; Amersham)}. The blots were hybridized at 68°C (nick-translated probes) or 60°C (Riboprobes) for 20 hr in a fresh aliquot of the above solution with 50% deionized formamide for Riboprobes only. The filters were then washed at 42°C for 2 hr in a buffer containing 50% deionized formamide/5× SSC/100 mM sodium phosphate and then were repeatedly washed in 0.1× SSC/0.1% $NaDodSO_4$ at 68°C. The filters were then exposed to Fuji RX or Amersham MP x-ray film with a Cronex intensifying screen (Du Pont) at −80°C.

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Abbreviation: IFN, interferon.

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S1 Mapping. Minus-strand probes for eight distinct IFN-α mRNAs were prepared from chromosomal IFN genes or cDNAs cleaved at an appropriate restriction site within the coding or 3′ untranslated regions of the gene (17). The probes were ^{32}P labeled at the 5′ terminus using polynucleotide kinase, cleaved at a second appropriate restriction site, and isolated from low-melting-temperature agarose as previously described (17). The IFN-α_1, -α_2, -α_4, and -α_{14} probes were derived from the following plasmid constructions: The *Pvu* II–*Bgl* II fragment (nucleotides 346–1750) of IFN-α_1 (18) was cloned into the *Hin*cII–*Bam*HI site of pSP 64 (15). The *Bgl* II–*Pst* I fragment (nucleotides 446–721) of IFN-α_2 (19) was cloned into the *Bam*HI–*Pst* I site of pSP 65. The *Bam*HI–*Hin*dIII fragment (nucleotides 1449–1649) of IFN-α_4 (6) was cloned into the *Bam*HI–*Hin*dIII site of pSP 65. The *Sau*3A–*Rsa* I fragment (nucleotides 1499–1839) of IFN-α_{14} (6) was cloned into the *Bam*HI–*Hin*cII site of pSP 65. IFN-α_1, -α_2, -α_4, and -α_{14} plasmids were cleaved with *Eco*RI (nucleotide 1564), *Ava* II (1693), *Hin*dIII (1649), and *Acc* I (1659), respectively; they were labeled at the 5′ terminus and digested with the restriction enzyme *Rsa* I, which cuts only within the vector DNA. The construction of the probes for IFN-α_5, -α_6, -α_7, and -α_8 has been described previously (17).

Each probe was hybridized with 40 or 400 μg of poly(A)$^+$ RNA from human organs or a known amount of the cognate RNA, which was synthesized *in vitro* using SP6 RNA polymerase (15, 16) in 40 or 400 μg of carrier RNA, and treated with S1 nuclease as previously described (17, 20). Following ethanol precipitation, the samples were resuspended in 50 mM Tris·HCl, pH 8.0/100 mM NaCl/5 mM EDTA (TNE) containing 2 μg each of denatured carrier DNA and carrier RNA treated with ribonuclease A (1 μg per 20 μg of RNA) and also ribonucleases T1 and T2 (1 unit per 10 μg of RNA) for 30 min at 37°C. The samples were then treated with proteinase K (100 μg/ml)/0.1% $NaDodSO_4$ for a further 30 min at 37°C, extracted with phenol/chloroform (1:1), and then applied to a column of Sephadex G-50 equilibrated with TNE. The fractions containing the protected probe were ethanol precipitated, resuspended in a gel loading buffer containing 90% deionized formamide, and applied to a 6.5% polyacrylamide/urea gel as previously described (20).

Quantitation of IFN mRNA in Uninduced Cells. The number of IFN mRNA transcripts per cell was estimated from the amount of total cellular RNA analyzed, the amount of RNA per cell (2×10^{-11} g) (21), the molecular weight of IFN mRNA (4×10^5), and the radioactivity of the protected DNA probe. The latter was determined relative to that given by a known amount of the cognate *in vitro* synthesized standard IFN RNA determined in parallel by densitometric scanning (transmission chromatogram analysis at 600 nm) of the autoradiograms using a Shimadzu scanner (model CS-930). The linear portion of the plot of peak area vs. IFN RNA (fmol) was used to quantitate samples.

RESULTS

Detection of IFN mRNA in the Organs of Normal Individuals. To determine if IFN messenger RNA could be detected in the organs of normal individuals, poly(A)$^+$ RNA was extracted from the spleens of accident victims whose organs had been taken for transplantation or from peripheral blood leukocytes from normal adult donors. The RNA was fractionated by electrophoresis in denaturing agarose gels, transferred to nylon filters, and hybridized to either cloned human IFN-α (18), -β (22), or -γ (23) genes labeled by nick-translation to a specific radioactivity of $\geq 2 \times 10^9$ cpm per μg of DNA or to Riboprobes of a similar specific activity. These techniques permitted the detection of as little as 0.3 attomole (amol) or 0.1 pg of *in vitro* synthesized specific IFN mRNA. A single distinct band of $\approx$1.2 kilobases that hybridized to the coding region of the human IFN-α_1 gene was detected in poly(A)$^+$ RNA isolated from the spleen and peripheral blood leukocytes of normal individuals (Fig. 1). This RNA comigrated with mature IFN-α mRNA from virus-induced leukocytes (Fig. 1). IFN-α mRNA was detected in all the samples of spleen tested from 12 normal donors, in the peripheral blood leukocytes of the four normal individuals tested, and in the one kidney and one liver tested from two different accident victims. No RNA hybridizing to human IFN-β_1 DNA was detected in any of the samples tested, even after extended periods of exposure of the autoradiograms under conditions where 0.3 amol of *in vitro* synthesized specific IFN-β_1 mRNA was readily detected after an overnight exposure (Fig. 2*A*). After hybridization with IFN-β_1 DNA, the RNA blots were dehybridized and rehybridized with a ^{32}P-labeled actin cDNA. A single strongly hybridizing band of 2.3 kilobases in length was observed after a 1-hr exposure of the autoradiograms, which demonstrates the integrity of the RNA (Fig. 2*B*).

Poly(A)$^+$ mRNA hybridizing to human IFN-γ cDNA was detected in one sample of human spleen (Fig. 3, lane 1), which also had an unusually high level of IFN-α mRNA but no IFN-β_1 mRNA (data not shown). The IFN-γ mRNA comigrated with mature IFN-γ mRNA from induced human peripheral blood lymphocytes (Fig. 3, lane 3). No IFN-γ mRNA was detected, however, in the five other normal spleens tested.

Characterization of Individual IFN-α RNA Species. To determine which IFN-α genes are expressed constitutively in normal human organs, we developed a modified S1 mapping procedure that permitted the detection and characterization of low levels of closely related IFN-α transcripts present in a large amount of unrelated RNA. Briefly, minus-strand probes for eight distinct IFN-α mRNAs were each hybridized with 40 or 400 μg of poly(A)$^+$ RNA from human organs and were treated with S1 nuclease. Then the probes that had been hybridized with 400 μg of poly(A)$^+$ RNA were also treated with pancreatic ribonuclease and ribonucleases T1 and T2. The IFN probe protected by the cognate RNA was then isolated from digested RNA by chromatography on Sephadex G-50 prior to loading on a polyacrylamide gel. This procedure

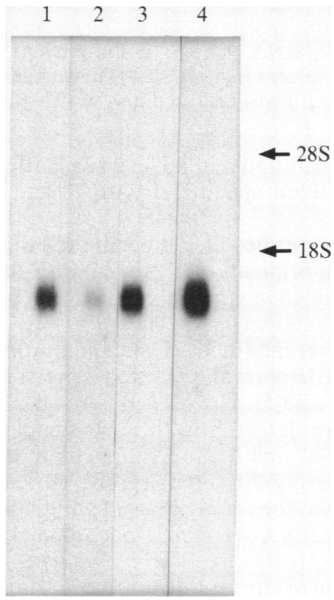

FIG. 1. Blot-hybridization analysis of IFN-α mRNA in the organs of normal individuals. Poly(A)$^+$ RNA from two human spleens (lanes 1 and 3), from human peripheral blood leukocytes (lane 2) or from human leukocytes induced with Newcastle disease virus (lane 4) were hybridized to cloned human IFN-α_1 DNA. Forty micrograms of RNA was loaded per sample except for Newcastle disease virus-induced human leukocytes where 1.5 μg of RNA was loaded per lane. The film was exposed for 7 days at −80°C.

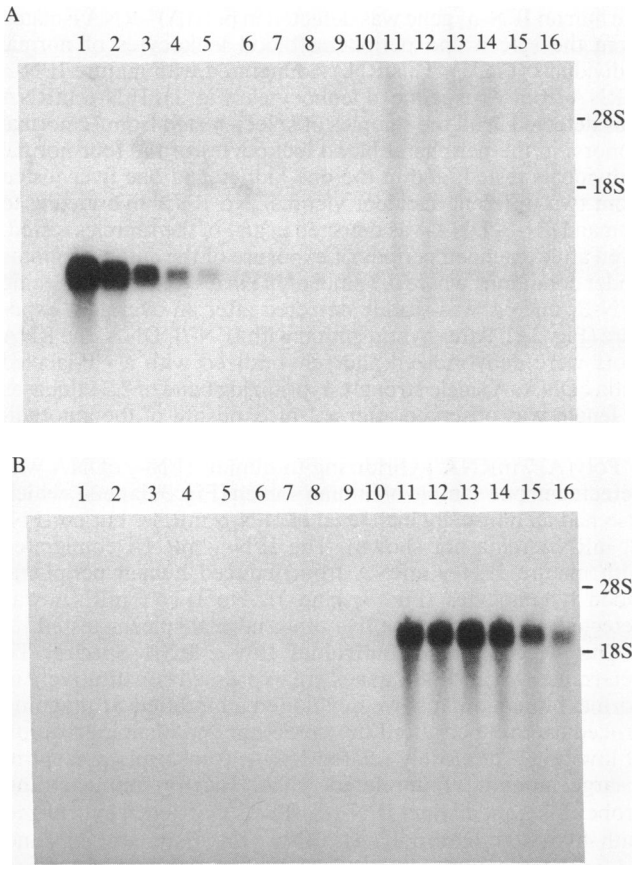

FIG. 2. Blot-hybridization analysis of IFN-β mRNA in the organs of normal individuals. (*A*) Hybridization to a human IFN-β_1 cDNA. The film was exposed for 24 hr at −80°C. (*B*) Hybridization to a mouse β-actin cDNA (24). The film was exposed for 1 hr at −80°C. Lanes 1–9: specific IFN-β_1 RNA synthesized *in vitro* using SP6 RNA polymerase (15) in 40 μg of carrier RNA; 0.1 fmol (lane 1), 0.03 fmol (lane 2), 0.01 fmol (lane 3), 0.003 fmol (lane 4), 0.001 fmol (lane 5), 0.3 amol (lane 6), 0.1 amol (lane 7), 0.03 amol (lane 8), 0.01 amol (lane 9), 40 μg of carrier RNA alone (lane 10). Poly(A)$^+$ mRNA (40 μg) from four human spleens (lanes 11–14), human kidney (lane 15), and human liver (lane 16) was analyzed in lanes 11–16.

permitted the quantitative recovery of as little as 0.01 fmol of *in vitro* synthesized specific IFN-α_2 RNA from 400 μg of carrier RNA.

All the IFN-α probes were specific for the cognate RNA and no cross-hybridization occurred with other IFN RNA species synthesized *in vitro* using SP6 RNA polymerase (ref. 17 and data not shown).

The use of this modified S1 mapping procedure revealed the presence of IFN-α_1 and -α_2 transcripts in normal human spleen (Figs. 4 and 5). No IFN-α_4, -α_5, -α_6, -α_7, -α_8, or -α_{14} transcripts were detected in the one sample of spleen that contained IFN-α_1 and -α_2 transcripts (data not shown). The fragments corresponding to the probe protected from the 3′ end of the gene to the restriction site within the coding region by the cognate RNA were 288 and 244 nucleotides long for IFN-α_1 and -α_2, respectively (Figs. 4 and 5). The fragments protected by the *in vitro* synthesized IFN-α_1 and -α_2 RNA are longer than those protected by natural mRNA, due to the presence of some vector sequences in the *in vitro* synthesized RNA. The fragment of ≈220 nucleotides detected in human RNA, in the *in vitro* synthesized RNA, and in the probe alone (Fig. 5) may result from a minor contaminant of the probe (46). IFN-α_1 and -α_2 transcripts were detected in all eight samples of normal human spleen tested and in the one sample each of human kidney and liver tested (Table 1). IFN-α_1 RNA

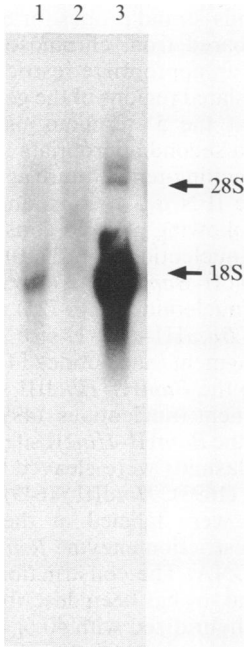

FIG. 3. Blot-hybridization analysis of IFN-γ mRNA in the organs of normal individuals. Poly(A)$^+$ RNA or poly(A)$^-$ RNA from human spleen (lanes 1 and 2, respectively) or Newcastle disease virus-induced leukocytes (lane 3), which have been shown to contain mature IFN-γ mRNA (17), was hybridized to a cloned cDNA of human IFN-γ (23). Twenty micrograms of RNA was loaded per sample. The film was exposed for 7 days at −80°C.

was present in human spleen and kidney at concentrations ranging from 0.001 to 0.1 fmol per 400 μg of poly(A)$^+$ RNA,

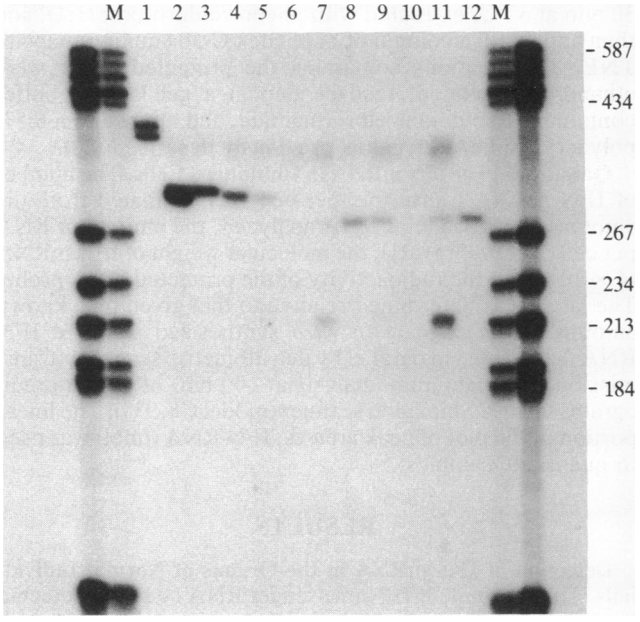

FIG. 4. S1 analysis of human IFN-α_1 transcripts in the organs of normal individuals. Poly(A)$^+$ RNA (400 μg) from the organs of normal individuals or a known amount of specific IFN-α_1 RNA synthesized *in vitro* using SP6 RNA polymerase (15) in 400 μg of carrier RNA was hybridized with 100 fmol each of a specific IFN-α_1 probe and treated with S1 nuclease. Lanes: M, size markers (pBR322 digested with *Bsp* I and 5′ ^{32}P-labeled); 1, IFN-α_1 probe alone; 2–6, IFN-α_1 RNA (lane 2, 0.1 fmol; lane 3, 0.03 fmol; lane 4, 0.01 fmol; lane 5, 0.003 fmol; lane 6, 0.001 fmol); 7, human spleen sample 3; 8, human spleen sample 5; 9, human kidney; 10, human liver; 11, human spleen sample 7; 12, human spleen sample 8. The film was exposed for 27 days at −80°C.

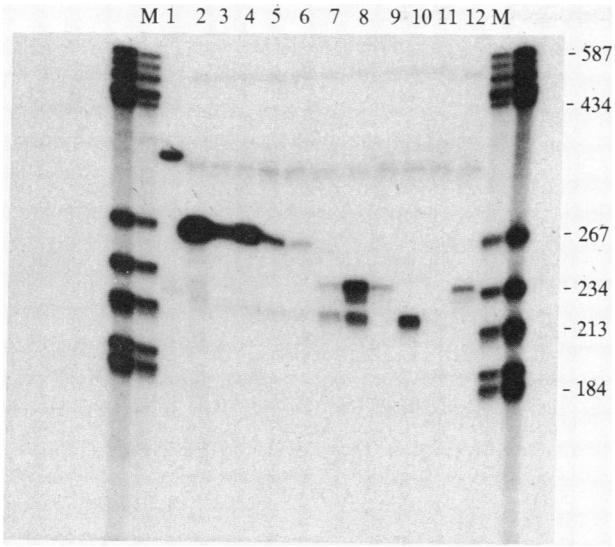

FIG. 5. S1 analysis of human IFN-α_2 transcripts in the organs of normal individuals. Poly(A)$^+$ RNA (400 μg) from the organs of normal individuals or a known amount of specific IFN-α_2 RNA synthesized *in vitro* using SP6 RNA polymerase (15) in 400 μg of carrier RNA was hybridized with 100 fmol each of a specific IFN-α_2 probe and treated with S1 nuclease as described. Lanes: M, size markers (pBR322 digested with *Bsp* I and 5′ ^{32}P-labeled); 1, IFN-α_2 probe alone; 2–6, IFN-α_2 RNA (lane 2, 0.1 fmol; lane 3, 0.01 fmol; lane 4, 0.03 fmol; lane 5, 0.003 fmol; lane 6, 0.001 fmol); 7, human spleen sample 3; 8, human spleen sample 5; 9, human spleen sample 6; 10, human kidney; 11, human liver; 12, human spleen sample 8. The film was exposed for 27 days at −80°C.

and IFN-α_2 was present at concentrations of 0.4–12 amol per 400 μg of poly(A)$^+$ RNA. In the single sample of normal liver tested, the concentration of both IFN-α_1 and -α_2 mRNA ($\approx$0.4 amol) was at the borderline of detection. The amount of IFN-α_1 mRNA present in normal human spleen was equivalent to at most 0.033 copy per cell and in some samples was as low as 3.0×10^{-4} copy per cell (Table 1), whereas that of IFN-α_2 varied from 1×10^{-4} to 4×10^{-3} copy per cell (Table 1).

DISCUSSION

We have shown that specific IFN-α genes are transcribed in the organs of normal individuals in the apparent absence of induction. The pattern of expression of IFN genes in normal human organs (only IFN-α_1 and -α_2 transcripts are detected) differs from that of virus-induced peripheral blood leukocytes or spleen cells, where IFN-α_1 and -β_1 are the major transcripts detected (refs. 16 and 17; M.G.T., unpublished results), or from that induced by platelet-derived growth factor or colony-stimulating factor, where only IFN-β is produced (25, 26). The absence of IFN-β_1 transcripts in normal human organs cannot be attributed either to an inherent weakness of the IFN-β_1 promotor since IFN-β_1 is expressed in high levels in many different cells following induction (16, 17) or to a lower sensitivity of detection since 0.3 amol of *in vitro* synthesized specific IFN-β_1 RNA can be detected under the experimental conditions used. The detection of a characteristic pattern of expression of IFN genes in all the samples tested together with the low number of transcripts produced per cell argue against these results simply reflecting opportunist virus infection or induction by growth factors and suggest that specific IFN genes are transcribed constitutively *in vivo*. Our results do not permit us to state, however, whether all the cells in a given population produce a low number of transcripts or a few cells are able to escape normal transcriptional controls and produce numerous copies of IFN mRNA.

Table 1. Number of IFN transcripts in the organs of normal individuals

Sample	Copies per cell × 10^3	
	IFN-α_1	IFN-α_2
Spleen		
1	33	0.39
2	36	0.18
3	0.33	0.36
4	1.0	0.11
5	1.4	3.6
6	—	0.51
7	1.0	—
8	1.7	0.84
Kidney	1.0	2.1
Liver	0.11	0.12

The number of IFN transcripts per cell was determined by densitometric scanning of autoradiograms obtained from S1 analysis of poly(A)$^+$ RNA from the organs of normal individuals.

These results have several important implications. First, such results challenge the concept that IFNs are produced *in vivo* solely in response to an infectious agent and may explain previous reports of the presence of an IFN-α-like substance in the amniotic fluid of pregnant women (9) and normal human bone marrow (10). Second, our results raise the possibility that the constitutive production of IFN *in vivo* could provide an important host defense against virus infection. Thus, our results could explain the long-recognized resistance of freshly explanted human peripheral blood monocytes to poliovirus infection and the decay of this antiviral state with time in culture (27). The importance of constitutive IFN production in host defense is also suggested by the results of studies with experimental animals, which indicate that IFN-α is also produced constitutively in the organs of normal mice (28) and that this IFN may play a role in host defense against both virus infection and neoplastic cells (11, 29, 30). Significant levels of the IFN-induced proteins 2–5′ oligo(A) synthetase (12, 31), p67K kinase (31), and Mx (32) have also been detected in the organs of normal uninfected mice, again suggesting that IFN is produced constitutively *in vivo*.

Although our results do not permit us to state whether or not IFN-α mRNA is actually translated *in vivo*, this seems a likely possibility in view of the biological data from both experimental animals and humans (9–11, 27, 29, 30). The amount of IFN-α mRNA present constitutively in normal spleen, although low compared to the 10 copies of IFN-α message present in human mononuclear cells following natural virus infection (17), would be sufficient, nevertheless, to exert significant localized biological activity. Several hundred molecules of IFN protein are produced per copy of IFN-α mRNA in normal human spleen cells (data not shown), and just a few molecules of IFN are sufficient to exert an antiviral activity (33).

Finally, our results demonstrate the remarkable precision with which IFN genes are regulated *in vivo*. IFN-α_1 and -α_2 genes are expressed at levels of $\leq$0.03 copy of mRNA per cell, whereas no IFN-β_1 message ($<1 \times 10^{-4}$ copy per cell) was detected in any of the samples tested even though a thousand or more transcripts of both genes can be detected within the same cell following induction (17). Thus, IFN genes appear to be subject to more stringent regulation than other inducible eukaryotic genes such as β-globin (34), histones (35), and metallothionein I (36), where the level of mRNA increases 10- to 100-fold following induction. The stringency of regulation of IFN genes is comparable to that of genes that code for proteins characteristic of a differenti-

ated tissue such as ovalbumin [in tissues such as liver where 0.01 copy per cell of mRNA are present constitutively (37)].

A gene designated IFN-β_2, which is structurally unrelated to other known interferon genes (38, 39) and has also been identified as a B-cell differentiation factor (40), has been described recently. The expression of this gene appears to be regulated in a manner quite different from that of other known IFN genes since IFN-β_2 mRNA is present endogenously in high levels in normal human spleen (M.G.T., unpublished results), which reflects either minimal transcriptional regulation or continued induction by cytokines such as interleukin 1 (41).

The use of a modified S1 mapping procedure with greatly enhanced sensitivity has allowed us to demonstrate constitutive expression of specific IFN genes *in vivo*. These techniques should be generally applicable to the study of other cellular genes. Studies using genomic sequencing (42) should determine whether the differential expression of IFN genes *in vivo* is regulated by mechanisms similar to those thought to control induction of IFN genes in cells in culture (4, 8).

In view of the known pleiotropic activities of IFN, our results raise the possibility that the constitutive production of IFNs *in vivo* may function to regulate such important physiological processes as fetal development, immune recognition, differentiation, hematopoiesis, and cell proliferation. Our results also provide the basis for the study of the expression of specific IFN genes in the tissues of patients with neoplasia or in patients with certain autoimmune or immune deficiency diseases characterized by an abnormal production of IFN (24, 43-45, 47).

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