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Induction of a mitochondrial matrix protein in human fibroblasts by interferon or poly I:C

The abundance of protein Mitcon: 5, a major component of monocytes and granulocytes as observed by high-resolution two-dimensional electrophoresis, is increased in human fibrolasts treated with interferon or poly I:C. Production of this polypeptide is eliminated in cells treated with dinitrophenol, but not in cells treated with chloramphenicol, indicating that it is a cytoplasmically synthesized mitochondrial protein. Neither chloroquine nor tunicamycin, both of which interfere with proper targeting of lysozomal enzymes, affects Mitcon:5 production. Actinomycin D prevents induction, indicating that new transcription is required. Cell fractionation studies show that Mitcon:5 is found in the mitochondria/lysosome fraction, and that it is released from this fraction by treatments designed to liberate matrix enzymes. Mitcon:5 thus appears to be the first example of a direct effect of interferon treatment on the composition of mitochondria. Further characterization of this effect may help to explain side-effects of interferon treatment, including fever.

1 Introduction

The interferons are proteins capable of inducing resistance to viral infection in a variety of cell types [1]. This effect is thought to arise at least in part because of the induction of specific enzymes, including an oligoadenylate synthetase [2] and a protein kinase [3]. Two-dimensional (2-D) electrophoretic analysis has, however, shown more than two proteins induced [4, 5], indicating that additional, unidentified proteins are likely to be involved in the response. Interferons alpha and beta induce a common set of polypeptides, while gamma interferon induces these and several more [6]. The study of these molecules is impeded by the general difficulty of characterizing polypeptides whose only known property is position on a two-dimensional (2-D) gel. At present, since most interferon-induced proteins are uncharacterized and unidentified, it is safe to say that the mechanisms of interferon's action in the cell are at least partially, perhaps largely, unknown.

In this paper, we have used information obtained in subcellular localization experiments, drug effect studies, and cell-type comparisons to characterize one of the prominent interferon-induced proteins of human fibrolasts. Such an approach is made possible by the use for all these studies of a reproducible 2-D gel system [7, 8], allowing accumulation of a database of results derived from a large series of experiments [9]. The protein concerned, designated Mitcon:5 in published maps of human white blood cells [10], has been of peripheral interest for some time. It is a member of the set of proteins whose production is stopped in cells treated with antimitochondrial agents [11], and thus is likely to be a cytoplasmically synthesized mitochondrial protein. Anti-mitochondrial agents such as nonactin, valinomycin or dinitrophenol eliminate the membrane potential across the mitochondrial inner membrane and thus prevent cleavage of cytoplasmically synthesized precursors into mature protein forms by the energy-dependent mitochondrial protein processing system [12]. Spots corresponding to the mature forms of mitochondrial proteins thus disappear from the pattern of cells treated with these agents; such proteins are termed Mitcon proteins [11]. Mitcon:5 is the most abundant such protein observable in monocytes and granulocytes in the pH range accessible with normal first-dimension isoelectric focusing gels, but is present only in trace quantities in lymphocytes and fibroblasts. It thus represents a protein strongly controlled in differentiation. Mitcon:5 is near the extreme basic end of the 2-D pattern and is apparently not resolved on most published 2-D patterns of human cells.

The discovery that Mitcon:5 is induced in fibroblasts by treatment with interferon [13] or poly I:C, a major interferon inducer [14], has encouraged us to try to characterize the protein further. Here we present the results of subcellular fractionation and drug treatment experiments designed to determine the submitochondrial location and site of synthesis of Mitcon:5. The results confirm the initial assignment as a mitocondrial polypeptide, and indicate that the protein is a soluble component located in the matrix, rather than the inner membrane. Mitcon:5 thus represents the first, and so far only interferon-induced protein known to be mitochondrial. A functional relationship between interferon and mitochondria could explain some of the side-effects of interferon treatment, such as fever [15].

2 Materials and methods

2.1 Cell culture

Human diploid fibroblasts (line 1494) were grown in 24-well microplates. Cells were labelled with 60 μCi/ml [^{35}S] methionine in RPMI 1640 medium lacking added methionine (Gibco Selectamine kit), supplemented with 10 % fetal bovine serum and antibiotics. For sample preparation, medium was removed by aspiration, and the cells solubilized by rinsing the well-bottom with 70 μl of 9 M urea, 4 % NP-40 detergent, 2 % carrier ampholytes (pH 9–11, LKB), 2 % mercaptoethanol. Treament compounds were added as 4 μl of 100 × stock solution to 400 μl total well volume. Poly I:C (Miles, 2 mg/ml), human fibroblast interferon (IFN beta, Calbiochem, 10^5 U/ml), chloroquine (2.5 mg/ml), tunicamycin (1 mg/ml), and dinitrophenol (DNP; 100 mM) stocks were made up in phosphate-buffered saline, and chloramphenicol (10 mg/ml) was prepared in ethanol.

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Abbreviations: **2-D**, two-dimensional; **MS**, mannitol-sucrose buffer; **DNP**, dinitrophenol

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2.2 Cell fractionation

A small tissue culture flask containing near-confluent fibroblasts was treated with 20 μg/ml poly I:C and concurrently labelled with 60 μCi/ml [^{35}S]methionine for 18 h. The labelled cells were removed from the flask with a rubber policeman and mixed with approximately 5 × 10^6 unlabelled human lymphoblastoid cells as carrier in a total of 200 μl MS buffer (200 mM mannitol, 50 mM sucrose, 10 mM HEPES pH 7.4, 1 mM EDTA, 0.05 % human serum albumin). This suspension was homogenized by passage through a 100 μl Hamilton syringe twice with rapid expulsion. A crude mitochondrial preparation was made with a Beckman TL-100 ultracentrifuge with a TLA-100 anglehead rotor holding 200 μl tubes and the following procedure (all at 0–4 °C): the sample was spun for 3 min at 5000 rpm (1100 *g*) and the pellet discarded (nuclei and unbroken cells). The supernatant was spun for 5 min at 15 000 rpm (10 000 *g*) and the supernatant (soluble fraction) discarded. The mitochondrial pellet was resuspended in mannitol-sucrose buffer (MS), and both low and high speed centrifugations repeated as above. The mitochondrial fraction was resuspended in MS plus 0.05 % digitonin and incubated on ice for 10 min. An aliquot was solubilized directly at this stage in urea/NP-40 as for whole cells ("whole mitochondria" sample). The remainder of the suspension was spun at 15 000 rpm for 10 min and the pellet (cleaned mitochondrial fraction minus outer membrane) taken up in 50 μl of MS plus 0.2 % Lubrizol WX [16] to disrupt the inner mitochondrial membrane. This suspension was spun for 20 min at 68 000 rpm (205 000 *g*), and pellet and supernatant were solubilized as "inner membrane" and "matrix" respectively. This procedure yields good fractionation starting with small amounts of material (200 μl cell suspension) and is extremely rapid due to the fast acceleration and deceleration of the TL-100 (with negligible stir-back of pelleted material).

2.3 Protein mapping

Ten to 20 μl samples prepared as above were analyzed with the 17 × 17 cm ISO-DALT 2-D gel system [7, 8]. Focusing gels contained wide-range (3–10) Bio-Rad carrier ampholytes, and second dimension gels were 9–16 % polyacrylamide gradient slabs cast by a computer-controlled pumping system ("Angelique"). Gels were dried and exposed to Kodak XAR2 film. Autoradiographs of duplicate gels of whole cells and cell fractions were analyzed with the TYCHO data reduction system [10].

3 Results

Eight major proteins induced in human fibroblasts by interferon β are shown in Fig. 1. These proteins are the same as those we have found to be induced in these cells by poly I: C. Mitcon: 5, a protein of about 20 000 Daltons, is one of the most abundant induced proteins in this pH range (about 4.5 to 7.8) in terms of incorporated methionine or total protein (Coomassie-stained gels, not shown). It has apparently not been observed in previous investigations of interferon's effects due to its relatively basic p*I*, at the edge of the pattern resolvable with an equilibrium isoelectric focusing first dimension. The vast majority of fibroblast proteins are not affected by interferon treatment. Mitcon: 5 is not induced in the human lymphoblastoid cell line GM607 by interferon or poly I: C, although many of the other interferon-inducible proteins shown here are induced (data not shown).

Fig. 2 shows the effect on interferon-induced Mitcon: 5 synthesis of a range of specific inhibitors. Chloramphenicol inhibits synthesis of mitochondrially encoded proteins, while dinitrophenol (DNP) stops production of both nuclear-encoded and mitochondrially encoded mitochondrial polypeptides [11]. Mitcon: 5 is not produced in poly I: C induced cells treated with DNP, demonstrating that appearance of the protein is dependent on the mitochondrial protein processing system and hence that this protein is likely to be mitochondrial. Chloramphenicol treatment, in contrast, does not affect Mitcon: 5 expression, demonstrating that the protein is not synthesized on mitochondrial ribosomes. Treatment of Mitcon: 5-producing cells with chloroquine [16] or tunicamycin [17] at levels found to substantially alter abundances of putative lysosomal proteins had no effect on Mitcon: 5 abundance (Fig. 2E, F). Concurrent treatment with interferon and actinomycin D stops induction of the interferon-inducible proteins, demonstrating that new transcription is required. Mitcon: 5 may thus be classified as a nuclear-encoded, cytoplasmically synthesized mitochondrial protein whose gene is caused to be transcribed in fibroblasts by interferon.

The abundance of Mitcon: 5 in a series of subcellular fractions is shown in Fig. 3, with associated quantitative data in Table 1. A large majority of the Mitcon: 5 of the cell is recovered in the mitochondrial fraction (> 70 %), while only small amounts of proteins known to be soluble (< 5 % of LDH-B, < 7 % of the major soluble heat shock protein) or cytoskeletal (< 5 % of the non-muscle tropomyosin) are found. When the mitochondria are gently broken by treatment with the detergent Lubrol WX [18], Mitcon: 5 is an abundant component of the supernatant matrix fraction, and is depleted in the pelleted inner-membrane fraction. A significant amount of the matrix protein remains in the inner-membrane fraction due to incomplete breakage of the mitochondria. Nevertheless, quantitative analysis (Table 1) demonstrates that the Lubrol technique separates mitochondrial proteins into two distinct classes, with known membrane proteins (one mitochondrially-encoded protein, Mitcon: 4, and the β-subunit of the F_1 ATPase, Mitcon: 1) appearing only in the inner membrane fraction, and a second class (matrix proteins) appearing in both fractions almost equally. Mitcon: 5 thus behaves as a mitochondrial matrix protein.

4 Discussion

The results presented here demonstrate that Mitcon: 5 behaves as a mitochondrial matrix protein whose production can be induced in human fibroblasts by interferon or poly I: C. The susceptibility of Mitcon: 5 expression to DNP treatment demonstrates that it is processed by the mitochondrial protein cleavage system during import into the mitochrondria, and the location of the protein in the mitochondrial/lysosomal cell fraction strengthens this conclusion even further. Insensitivity to the effects of chloramphenicol (which prevents translation on mitochrondrial ribosomes) demonstrates that Mitcon: 5 is not mitochondrially encoded and translated. Chloramphenicol treatment does interrupt production of Mitcon: 4 (Mitcod: 1), a hydrophobic protein likely to be subunit II of cytochrome oxidase, indicating that the drug works under these conditions

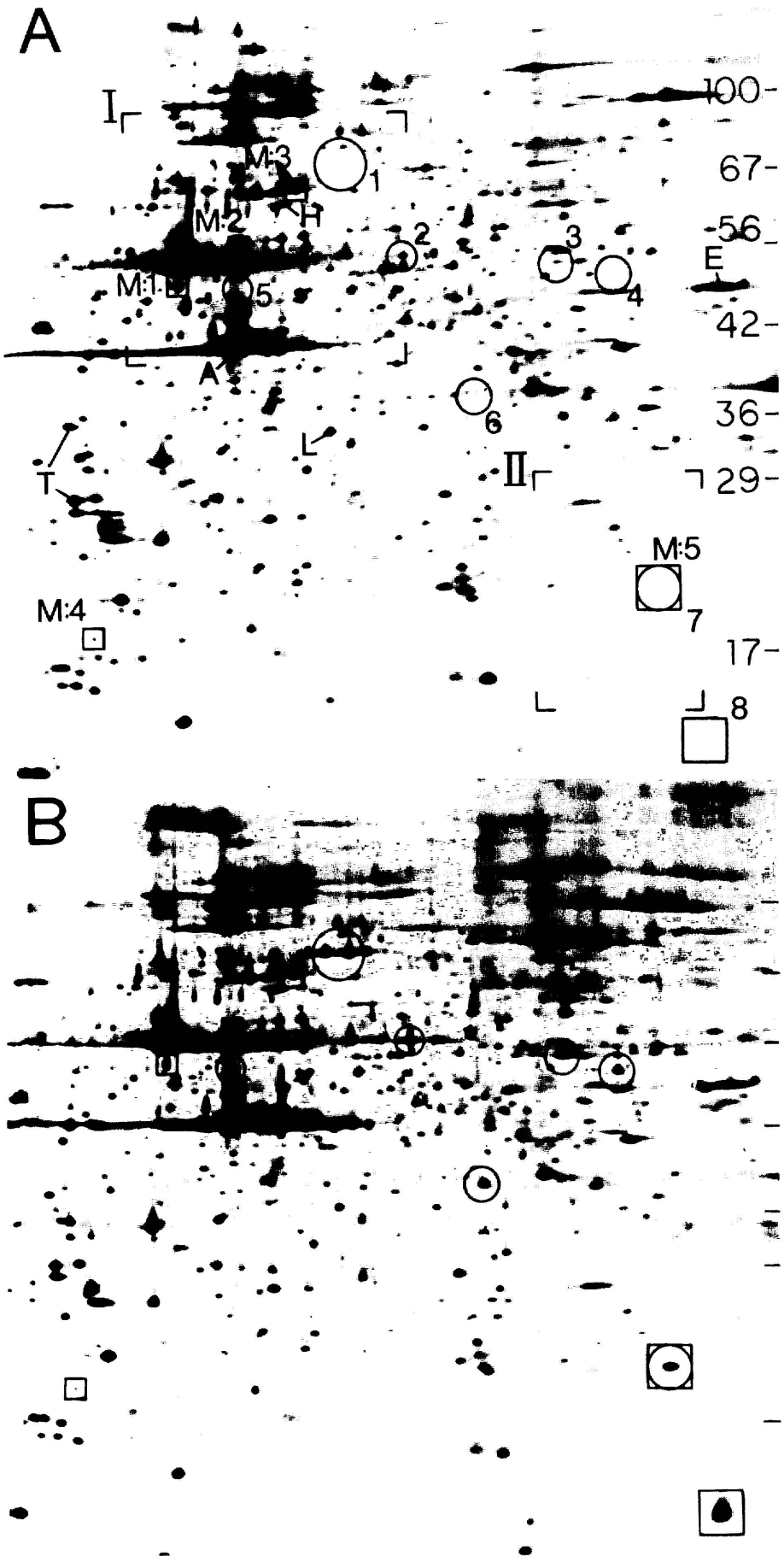

Figure 1. Two-dimensional electrophoretic pattern of proteins (A) from the normal human fibroblast line 1494, and (B) from the same line treated with 1000 U/ml human fibroblast interferon. Circles enclose eight prominent interferon-induced proteins (Interf:1-Interf:8). Squares enclose prominent mitochondrial proteins, labelled M:1–5 for Mitcon:1-Mitcon:5 [11]. Protein Interf:7 is identical to protein Mitcon:5, a major protein of human monocytes and granulocytes. Mitcon:1 is the beta-subunit of the mitochondrial F1-ATPase, while Mitcon:4 is a mitochondrially encoded and synthesized protein, probably the subunit II of cytochrome oxidase. A is actin, L is LDH-B, H is the major heat shock protein, and T indicates two of the several forms of tropomyosin found in fibroblasts (smooth muscle TM:3 above and a non-muscle TM:4 below). Two regions examined more closely in remaining figures (I and II) are bracketed. Approximate sodium dodecyl sulfate molecular weights are indicated along the right side of (A).

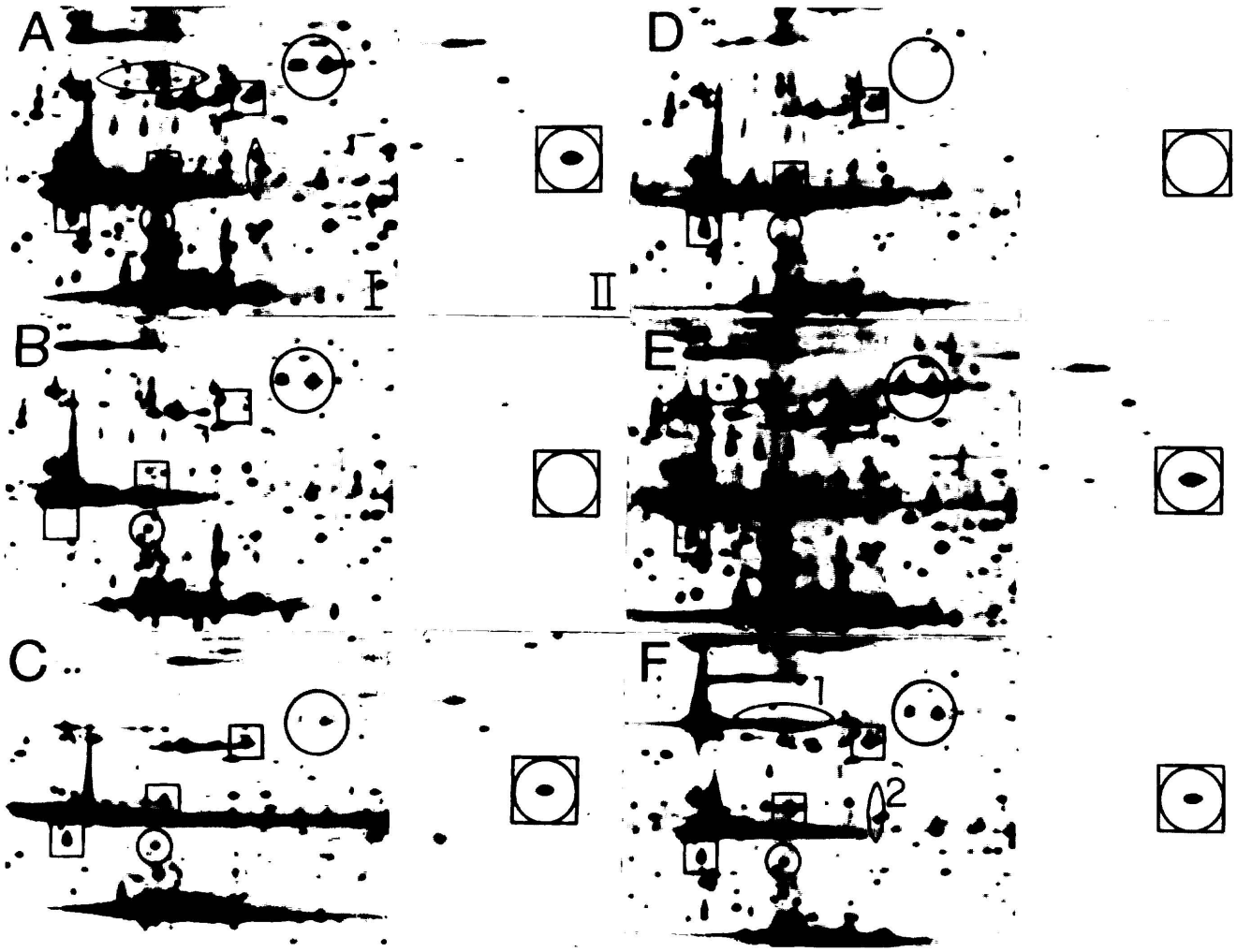

Figure 2. Montage showing two regions (I and II indicated on Fig. 1A) of each of six gels (A–F). (A) shows fibroblasts plus 20 μg/ml poly I:C (same proteins as those induced by interferon), (B) fibroblasts plus poly I:C and 1 mM dinitrophenol (Mitcon proteins removed due to inactivation of the mitochondrial protein processing system), (C) fibroblasts plus poly I:C and 100 μg/ml chloramphenicol (no effect on Mitcon:5/Interf:7), (D) fibroblasts plus 1000 U/ml interferon and 10 μg/ml actinomycin D (no interferon-inducible proteins expressed), (E) fibroblasts plus interferon and 25 μM chloroquine (no effect on interferon inductions), and (F) fibroblasts plus interferon and 10 μg/ml tunicamycin (no effects on Mitcon:5 induction, but several other effects indicated by ovals, interpreted as disappearance of glycosylated version of normal glycoproteins, and appearance in some cases of non-glycosylated versions; see vertical shift of spot in oval 2).

Table 1. Quantitative analysis of subcellular localization[a)]

Protein	Production in: Fibroblasts + Poly I:C	Whole mitochondria	Mitochondrial membrane	Mitochondrial matrix
Mitcon:1	24790	34805	43756	< 500
Mitcon:2	29538	37017	30940	32956
Mitcon:3	31696	24416	30431	28424
Mitcon:4	4455	7307	8387	< 500
Mitcon:5	77239	55704	45920	52743
LDH-B	18335	< 500	< 500	< 500
Heat-shock:1	17421	1138	< 500	< 500
Non-muscle Tm	52283	2382	< 500	< 500

a) Each value is the average of the integrated densities on two gels of the same sample. Images were analyzed with the TYCHO 2-D analysis system as described [10]. The different gels were roughly scaled together by setting the amount of Mitcon:2 + Mitcon:3 equal for each gel. Spots that were undetectable are given the value < 500, 500 being the smallest spot reliably detectable in this series.

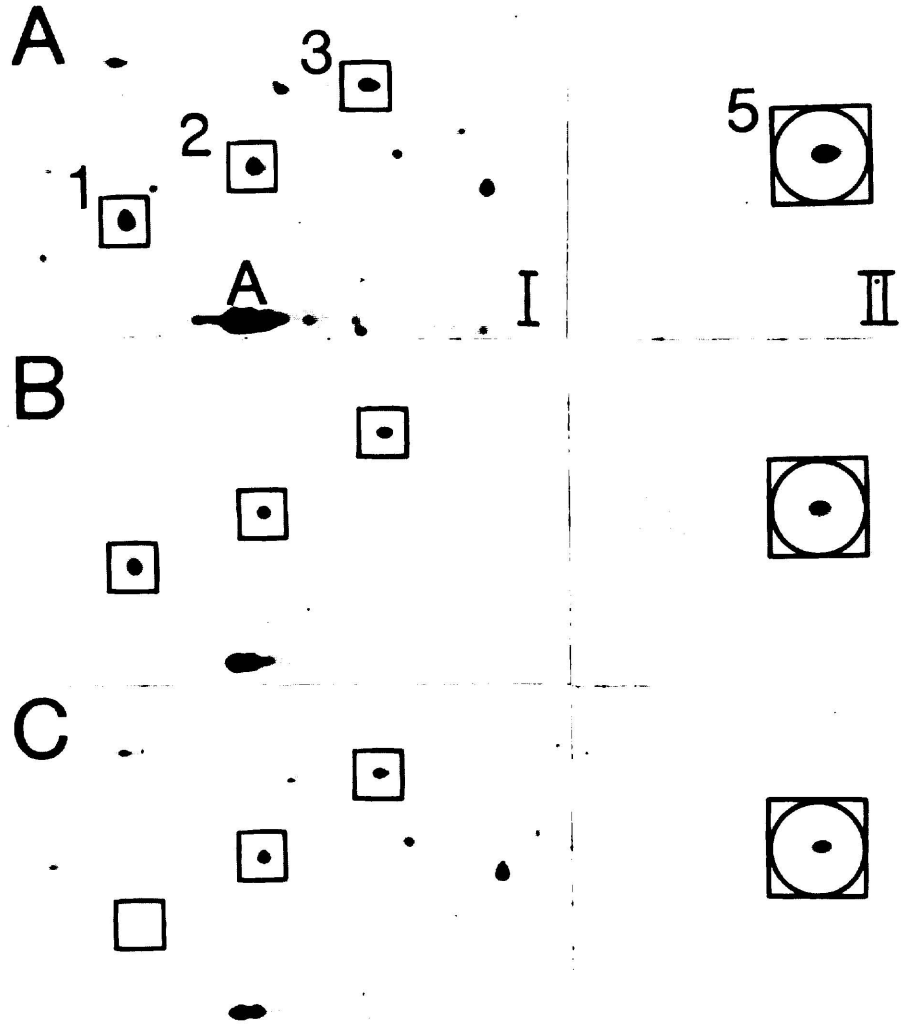

Figure 3. Montage showing regions I and II of gels of: (A) whole mitochondria, (B) mitochondrial inner membrane fraction, and (C) the mitochondrial matrix fraction released by Lubrol WX. Quantitative results from these gels are shown in Table 1.

(data not shown). This feature is of interest because of the observation [19] that a large proportion of cloned interferon-induced sequences were homologous to mitochrondrial ribosomal RNA sequences. Possibly the presence in the mitochondrion of an abundant interferon-induced protein results in increased transcription of the mitochondrial ribosomal RNA genes.

Mitcon: 5 is not an interferon-specific protein, in the sense that it is normally expressed at high levels in monocytes and granulocytes, cells with important functions in the defense against infectious diseases. Few cultured cell lines produce Mitcon: 5 in greater than trace amounts; the exceptions observed so far include cells (such as HL-60 treated with phorbol esters) that are differentiating into monocyte/macrophages, and fibrobasts treated with interferon or inducers. It may therefore be suggested that Mitcon: 5 is a matrix enzyme associated with some metabolic function related to defense at the cellular level. One aspect of this function might be the generation of excess heat giving rise to the "interferon fever" often observed as a side effect of interferon treatment. A detailed explanation of the significance of Mitcon: 5 induction awaits the identification of its enzymatic (or other) activity.

From the viewpoint of gene regulation, it is perhaps surprising that interferon can induce synthesis of Mitcon: 5 in fibroblasts but not in lymphoid lines that are in some sense closer to the monocytes and granulocytes which express it continuously. A detailed study of the responses of a variety of cell types to each of the three interferons (α, β, γ) may help solve this puzzle.

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