Interferons induce xanthine dehydrogenase gene expression in L929 cells

Francesco FALCIANI,* Pietro GHEZZI,† Mineko TERAOKA,* Giovanni CAZZANIGA* and Enrico GARATTINI*‡

*Molecular Biology Unit, Centro Daniela e Catulla Borgomainerio and †Laboratory of Neuroimmunology, Istituto di Ricerche Farmacologiche 'Mario Negri', via Eritrea, 62, 20157 Milano, Italy

Human interferon- α A/D (*BgIII*) (IFN- α A/D) and mouse interferon- γ (IFN- γ) are shown to induce xanthine dehydrogenase (XD) mRNA in L929 fibroblastic cells. XD mRNA accumulation after IFN- α A/D treatment is relatively fast, being already evident after 4 h and reaching its maximum after 24 h. IFN- α A/D is active in inducing XD mRNA at 0.1 unit/ml and it is maximally active at 10^3 units/ml. The half-life of the XD message is unaffected by IFN- α A/D treatment, whereas the transcriptional activity of the XD gene and the concentrations of XD heterogeneous nuclear RNA are increased by 2- and 6-fold respectively. The effect of IFN- α A/D on XD mRNA is insensitive to cycloheximide, suggesting that protein synthesis *de novo* is not required. Experiments conducted with specific inhibitors suggest that protein kinase C, cyclic AMP and arachidonic acid metabolites derived from lipoxygenase or cyclooxygenase do not act as second-messenger molecules in the induction of XD mRNA by IFN- α A/D. XD mRNA is also induced in NIH3T3 fibroblastic cells, but not in F9 teratocarcinoma or B16 melanoma cells after treatment with IFN- α A/D. NIH3T3 are the only cells so far tested that have detectable XD and xanthine oxidase activities under basal conditions and after IFN- α A/D treatment, although their responsiveness to the cytokine is much less than that observed in L929 cells.

INTRODUCTION

Interferons (IFNs) have pleiotropic effects on various cell types: they induce an antiviral state, they generally inhibit the proliferation of both normal and tumour cells and are involved in the complex network of cytokines that regulates the haematopoietic system (Isaac & Lindenmann, 1957; Lengyel, 1982; Kirchner & Shellekens, 1984; Pestka *et al.*, 1987). There are three major antigenic groups of IFNs, i.e. α , β and γ . The former two are also known as type-I IFNs whereas the latter is known as type II (Pestka *et al.*, 1987). IFNs modulate the expression of more than thirty genes in various cell types (Staeheli, 1990), and most of these genes are of unknown function.

IFNs- α/β and IFN- γ act through two distinct membrane receptors (Aguet, 1991) and regulate the expression of different but partly overlapping subsets of genes (Weil *et al.*, 1983; Benech *et al.*, 1985a; Boss & Strominger, 1986; Reich *et al.*, 1987; Luster & Ravetch, 1987; Porter *et al.*, 1988; Reid *et al.*, 1989). Recombinant human interferon- α A/D (*BgIII*) (IFN- α A/D), a type-I IFN that crosses animal species barriers, is capable of inducing mouse xanthine oxidase (EC 1.1.3.22; XO) and xanthine dehydrogenase (EC 1.1.1.204; XD) activities *in vivo* (Ghezzi *et al.*, 1984, 1985). The two enzyme activities are involved in the intracellular catabolism of purines and carry out the same metabolic steps, i.e. transformation of hypoxanthine into xanthine and xanthine into uric acid. However, whereas XO transfers the reducing equivalents generated by the enzyme reactions to molecular oxygen, XD transfers them to NAD⁺. The two enzyme forms are the products of a single gene (the gene and the transcript coding for XD and XO are referred to as the XD gene and XD transcript respectively throughout this paper) and they are interconvertible in several experimental conditions both *in vitro* and *in vivo* (Della Corte & Stirpe, 1968, 1972).

XO activity has been suggested to play a role in the hepato-

toxicity observed *in vivo* after IFN treatment, probably because of its capacity to produce highly reactive superoxide anions. In fact, IFN- α A/D as well as bacterial lipopolysaccharide (LPS) and polyriboinosinic:polyribocytidylic acid (poly I/C) induce XO activity and depress cytochrome *P*-450 concentrations in mouse liver (Ghezzi *et al.*, 1984, 1985; Carpani *et al.*, 1990). The effect of IFN *in vivo* is prevented by pretreatment of animals with allopurinol and *N*-acetylcysteine, a specific inhibitor of XO and a scavenger of oxygen radicals respectively (Ghezzi *et al.*, 1985). It is also possible that XO (or XD) is involved in the antiviral, antiproliferative or immunomodulatory activity of IFNs.

The cDNA coding for mouse XD has been cloned in our laboratory, allowing us to demonstrate that the induction of XO activity in the liver by IFN is primarily the consequence of an increased accumulation of XD mRNA and it is not due to an increased conversion of XD into XO (Terao *et al.*, 1992). To study in more detail the molecular mechanisms underlying the induction of the expression of the XD gene, experiments were carried out using L929 fibroblastic cells, because they are widely used *in vitro* as a model system for the antiviral effect of IFNs. The data presented in this report suggest that the XD gene represents a primary target of both type-I and type-II IFNs. Furthermore the increased accumulation of the XD transcript after treatment with type-I IFNs is the result of an induction in the transcriptional rate of the gene as well as early post-transcriptional nuclear events.

MATERIALS AND METHODS

Cell lines and reagents

L929 is a fibroblastic cell line obtained from the American Type Culture Collection (A.T.C.C.), Rockville, MD, U.S.A. These cells were routinely passaged in RPMI 1640 containing 10% (v/v) fetal-calf serum. F9 teratocarcinoma cells (from Dr.

Abbreviations used: IFN, interferon; XD, xanthine dehydrogenase; XO, xanthine oxidase; LPS, lipopolysaccharide; poly(I/C), polyriboinosinic:polyribocytidylic acid; $1 \times \text{SSC}$, 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0; oligoAS, oligoadenylate synthetase; ISRE, IFN-stimulated responsive element.

‡ To whom correspondence and reprint requests should be addressed.

B. Terrana, Sclavo Laboratories, Siena, Italy), NIH3T3 fibroblastic cells and B16 melanoma cells (from A.T.C.C.) were grown in Dulbecco's modified Eagle's medium containing 10% fetal-calf serum. Cells were seeded at a concentration of 10^5 cells/ml in 25 or 75 cm² Falcon culture flasks (Becton-Dickinson, Lincoln Park, NJ, U.S.A.) and allowed to attach to the plastic substrate for 4–6 h before the appropriate treatment was performed. Cultures were free from mycoplasma, as assessed using the Hoechst 33258 fluorescent-dye system (Farbwerke Hoechst AG, Frankfurt, Germany). Recombinant human interferon- α A/D (6.4/10⁶ units/mg) was a gift from Dr. M. Brunda (Hoffmann-La Roche, Nutley, NJ, U.S.A.). Bacterial LPS, poly(I/C), cycloheximide, actinomycin D, phorbol 12-myristate 13-acetate, dibutyryl cyclic AMP and staurosporine were purchased from Sigma (St. Louis, MO, U.S.A.). The protein kinase C inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7) was from Seikagaku Inc., Tokyo, Japan.

Northern-blot analysis

Total RNA was prepared from cells according to a modification of the guanidinium isothiocyanate/caesium chloride method (Rambaldi *et al.*, 1987). Nuclear RNA was extracted from cell nuclei isolated by sucrose-gradient centrifugation (Marzluff & Huang, 1984). The RNA (10 or 15 μ g) was then fractionated on a 1.2% agarose gel with 6% formaldehyde and blotted on to synthetic nylon membranes (GeneScreen Plus; New England Nuclear, Boston, MA, U.S.A.). These membranes were hybridized with XDgt1, a 1.8 kb *Eco*RI fragment of mouse liver XD cDNA (Terao *et al.*, 1992), mouse α -actin cDNA (Minty *et al.*, 1981) or histone H2a cDNA (Seiler-Tuyns & Birnstiel, 1981). The probes were labelled to a specific radioactivity of 1×10^9 – 2×10^9 c.p.m./ μ g by using hexanucleotide primers and [³²P]dCTP (Amersham, Little Chalfont, Bucks., U.K.) (Feinberg & Vogelstein, 1983). Hybridization was performed at 60 °C overnight in a solution containing 1 M-NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate (Sigma), 100 μ g of salmon sperm DNA/ml (Boehringer, Mannheim, Germany) and 1×10^6 – 2×10^6 c.p.m. of radiolabelled probe/ml. The membranes were washed twice with $2 \times$ SSC/1% (w/v) SDS ($1 \times$ SSC being 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0) for 1 h at 65 °C and $0.1 \times$ SSC for 30 min at room temperature. The membranes were dried and exposed to Kodak-XOMat X-ray films with two intensifying screens (du Pont Cronex, du Pont de Nemours, Bad Homburg, Germany) at –70 °C.

Nuclear transcription run-on assay

Nuclear transcription run-on assays were performed as described by Greenberg & Ziff (1984) with some modifications. Briefly, nuclei were prepared by lysing cells (approx. 1×10^7) with 4 ml of lysis buffer [0.5% (w/v) Nonidet P40/0.01 M-NaCl/0.003 M-MgCl₂/0.01 M-Tris, pH 7.4]. After being washed with ice-cold lysis buffer, nuclei were resuspended in glycerol buffer [40% (w/v) glycerol/0.005 M-MgCl₂/0.0001 M-EDTA/0.05 M-Tris, pH 8.0] and incubated at 30 °C for 30 min in run-on buffer containing 0.005 M-Tris, pH 8.0, 0.0025 M-MgCl₂, 0.15 M-KCl, 0.00125 M each of ATP, CTP, GTP (Pharmacia, Uppsala, Sweden) and 100 μ Ci of [³²P]UTP (Amersham). Nuclei were then resuspended in 4 M-guanidinium isothiocyanate, and nascent RNA was recovered by centrifugation through caesium chloride and ethanol precipitation. Labelled elongated RNAs (minimum 1×10^6 c.p.m./ml) were hybridized to 5 μ g each of the plasmid cDNAs immobilized on nitrocellulose membranes after denaturation by heat and alkaline treatments. The filters were washed in $0.2 \times$ SSC at 65 °C for 30 min and then treated with 1 μ g of RNAase A/ml (Sigma) in $0.2 \times$ SSC for 30 min at room temperature. The cDNAs used for these experiments were:

mouse liver XD cDNA (XDgt1; Terao *et al.*, 1992), histone H2a cDNA (Hatch & Bonner, 1988) and α -actin cDNA (Minty *et al.*, 1981).

Autoradiograms of both Northern-blot analysis and nuclear run-on assays were quantified by laser-scanning densitometry with a laser beam densitometer (300 A computing densitometer Fast Scan; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Measurement of XD and XO activities

Cell monolayers (approx. 4×10^6 cells) from a 25 cm² dish were washed twice with 0.9% NaCl, harvested using a 'rubber policeman', and pelleted by centrifugation at 1500 rev./min. Cells were resuspended in 60 μ l of homogenization buffer (0.05 M-Tris/HCl, pH 7.8) and disrupted by sonication using a Branson sonifier at its maximum setting, twice, for 5 s at 4 °C. The total homogenate (3 μ l) was used for XD and XO assays, using [8-¹⁴C]hypoxanthine (Amersham) as substrate by the procedure of Reiners *et al.* (1987). XO and XD activities were normalized for the content of protein in the sample. Proteins were measured by the method of Bradford (1976) with BSA as standard. One unit is defined as the amount of enzyme capable of transforming 1 nmol of substrate into xanthine and uric acid in 1 min at 37 °C.

RESULTS

L929 fibroblastic cells were chosen to study the molecular mechanisms underlying the induction of the XD mRNA by interferons (IFNs) *in vitro*, because these cells are widely used to assay the antiviral effect of type-I IFNs and because they are known to express both IFN- α/β and IFN- γ receptors. Fig. 1(a) shows that IFN- α A/D is capable of inducing XD mRNA [the size of this transcript is similar to that of ribosomal 28S RNA, as expected from our previous work (Terao *et al.*, 1992)] after 18 h of incubation, whereas the concentrations of α -actin mRNA are not changed relative to control. The induction of the specific transcript is variable and ranges between 7- and 13-fold according to the experiment. Treatment of L929 cells with LPS or poly(I/C), two inducers of IFN activity, does not result in the induction of XD mRNA, contrary to what is observed *in vivo* (Terao *et al.*, 1992). The production of IFN by L929 cells is thus probably too low to determine activation of the expression of the XD gene in these experimental conditions or these cells do not express receptors for LPS or double-stranded RNA. As shown in Fig. 1(b), after 18 h of treatment, both interferon- α A/D (*Bg*III) (IFN- α A/D) and interferon- γ (IFN- γ) induce the accumulation of XD mRNA to similar levels. Moreover, poly(I/C) does not show any synergistic effects with either IFN- α A/D or IFN- γ . In order to compare the pattern of induction of XD with that of a classical marker for the biological activity of IFNs, the blots were rehybridized with an oligoadenylate synthetase (oligoAS) cDNA. This cDNA hybridizes with a low- and a high-*M_r* mRNA species (Benech *et al.*, 1985b). Only data regarding the low-*M_r* oligoAS mRNA, migrating as a 1600-nucleotide-long transcript, are presented throughout this report, since the higher-*M_r* mRNA behaves in a similar manner. The induction of oligoAS is always parallel with that of XD mRNA even though the latter transcript is much more sensitive to the inducing effect of IFN- γ in the experimental conditions presented in Fig. 1(b).

As shown in Fig. 2(a), the time-course for the induction of XD mRNA by IFN- α A/D is quite rapid, since an increase in the levels of the transcript is already evident after 4 h and reaches its maximum between 24 and 48 h. Moreover, the induction of XD mRNA is observed for all the concentrations of the cytokine used, as shown in Fig. 2(b). The maximal induction is, however, attained between 10^3 and 10^5 units/ml, which is similar to that observed for oligoAS. A similar time-frame is again observed for

Fig. 1. Effect of interferons and interferon inducers on XD mRNA accumulation in L929 cells

Total RNA (20 μ g for each lane) was extracted from L929 cells incubated for 18 h in medium alone (control) or in medium containing the indicated compound(s). The positions of the size markers (28S and 18S rRNA) are indicated. IFN, interferon- α A/D (10³ units/ml); IFN- γ , interferon- γ ; LPS, bacterial lipopolysaccharide (10 μ g/ml); Poly(I/C), polyriboinosinic/ribocytidylic acid (15 μ g/ml). (a) The same filter was sequentially hybridized with XD and α -actin cDNAs. (b) The same filter was used for hybridization with XD, oligoAS and α -actin cDNAs.

Fig. 2. Effect of the exposure time and concentration of IFN- α A/D on the induction of XD mRNA in L929 cells

(a) Cells were treated with IFN- α A/D for the indicated time. RNA was extracted and used for Northern-blot analysis (20 μ g/lane). The same filter was rehybridized sequentially with XD, oligoAS and α -actin cDNAs. (b) Cells were treated with the indicated amount of IFN- α A/D (IFN) for 18 h before RNA extraction. Northern-blot analysis was conducted as in (a). (c) Cells were treated with IFN- α A/D for the indicated time, washed and incubated with fresh medium without the cytokine for up to 18 h. RNA was extracted and used for Northern-blot analysis as in (a). The positions of the size markers (28S and 18S rRNA) are indicated.

the induction of the oligoAS transcript. To assess whether the continuous presence of IFN- α A/D is required for XD mRNA induction, L929 cells were cultured in the presence of the cytokine

for different amounts of time and XD transcript levels were measured at 18 h. Fig. 2(c) demonstrates that IFN- α A/D needs to be in continuous contact with L929 cells for maximal induction

Fig. 3. Effect of cycloheximide and actinomycin D on the induction of XD mRNA by IFN- α A/D

L929 cells were cultured in the absence (control) or presence of IFN- α A/D (IFN) in medium alone or in medium containing 10 μ g of cycloheximide (CHX)/ml or 5 μ g of actinomycin D (Act D)/ml. Total RNA was extracted and used for Northern-blot analysis (20 μ g/lane). The same filter was sequentially hybridized with XD, oligoAS and α -actin cDNAs. The positions of the size markers (28S and 18S rRNA) are indicated.

Fig. 4. Effect of IFN- α A/D on the transcription of the XD gene

Nuclear run-on assays were performed at the indicated times after incubation of L929 cells in the presence of 10^3 units of IFN- α A/D/ml. The probes used in this experiment are indicated. Plasmids pBR322 and p106 were used as negative controls. Actin, α -actin cDNA; histone, histone H2a cDNA.

of XD mRNA at 18 h, whereas 10 min of contact between the cells and IFN- α A/D are enough to trigger a maximal response of oligoAS mRNA at 18 h.

Fig. 5. Effect of IFN- α A/D on the stability of XD mRNA

Cells were cultured in the absence (control) or presence of IFN- α A/D (IFN). After 18 h, fresh medium containing 10 μ g of actinomycin D/ml was added. Total RNA was extracted at the indicated times after actinomycin D addition and 20 μ g was used to perform Northern-blot analysis. The same filter was sequentially hybridized with XD, histone H2a and α -actin cDNAs. The positions of the size markers (28S and 18S rRNA) are indicated.

As shown in Fig. 3, the induction of XD mRNA by IFN- α A/D does not require protein synthesis *de novo*, since the accumulation of the transcript is not inhibited by cycloheximide. In contrast, cycloheximide superinduces XD mRNA (about 1.5-fold) in both the absence and presence of IFN- α A/D. The effect is similar to that observed for oligoAS, even though the superinduction of this latter transcript after IFN- α A/D challenge is more evident (at least 10-fold). Treatment of cells with actinomycin D, a known inhibitor of RNA synthesis, completely blocks the induction of both XD and oligoAS mRNAs by IFN- α A/D.

Since the inhibitory effect of actinomycin D suggests a transcriptional mechanism, nuclear run-on experiments in the absence and presence of IFN- α A/D were performed using XD, oligoAS, actin and histone H2a cDNAs, and the results are shown in Fig. 4. As expected, the transcriptional activity of the genes for actin and histone H2a is not affected by treatment with IFN- α A/D at all the time-points tested, whereas the cytokine induces the transcriptional activity of the XD gene 2.4-fold by 2 h. Furthermore, nuclear run-on signals for XD remain constantly elevated at least up to 6 h (by 2–2.5-fold at all the time-points tested) after IFN- α A/D treatment. The transcriptional activity of the oligoAS gene is also increased with a similar time-frame. However, owing to its lower level of transcription under basal conditions, the induction of the run-on signal after IFN- α A/D treatment is quantitatively more evident (at least 10-fold). Run-on experiments were repeated twice with consistent results. The elevation in the transcriptional activity for both oligoAS and XD genes precedes the increase in the respective mRNA accumulation (compare Fig. 4 with Fig. 2a).

To determine whether the IFN- α A/D-mediated effects on XD mRNA were due to alterations in the stability of the XD message, we examined the effects of the cytokine in the presence of actinomycin D. As shown in Fig. 5, the half-life of the XD

Fig. 6. Effect of IFN- α A/D on the accumulation of XD heterogeneous nuclear RNA

Cells were cultured in the absence (control) or presence of 10^8 units of IFN- α A/D (IFN)/ml for 18 h. Total RNA (T) or nuclear RNA (N) was extracted from the same culture dishes and used for Northern-blot analysis ($20\text{ }\mu\text{g/lane}$). The positions of the size markers (28S and 18S rRNA) are indicated.

message is not significantly altered after treatment with IFN- α A/D (about 12 h and 16 h before and after treatment with the cytokine respectively). The decay curve for a short-lived message (histone H2a) and for a long-lived one (α -actin) are also shown for comparison. For these two mRNAs we calculated half-lives of 4 and more than 18 h respectively.

Because the XD transcriptional activity and mRNA decay kinetics were not altered concomitantly with changes in steady-state XD mRNA levels, we sought to determine whether the changes in XD mRNA are caused by a nuclear non-transcriptional event. To test this possibility, we purified nuclear and cytoplasmic (unfractionated or total RNA is mainly constituted of cytoplasmic RNA) steady-state RNA from L929 cells cultured in the absence or presence of IFN- α A/D for 18 h. Densitometric analysis of the blots shown in Fig. 6 demonstrates that the steady-state levels of XD heterogeneous nuclear RNA are increased about 6-fold after IFN- α A/D treatment, under conditions where the cytoplasmic XD transcript is induced about 4-fold. Notice that the lower than normal level of induction of XD mRNA is a consequence of the fact that L929 cells were treated at confluence to obtain enough nuclei for the extraction of heterogeneous nuclear RNA. Under these experimental conditions, XD mRNA accumulation is less after IFN- α A/D treatment (F. Falciani, P. Ghezzi, M. Terao, G. Gazzaniga & E. Garattini, unpublished work).

In order to investigate possible second-messenger molecules involved in IFN- α A/D induction of XD messenger RNA in L929 cells, we focused our attention on protein kinase C and cyclic AMP since both systems have been suggested to mediate some of the biological effects of IFNs (Schneck *et al.*, 1982; Nagata *et al.*, 1984; Reich & Pfeffer, 1990). The results shown in Fig. 7(a) demonstrate that protein kinase C does not play any role in the induction of XD mRNA by IFN- α A/D. In fact, phorbol 12-myristate 13-acetate, a general direct activator of protein kinase C is neither capable of inducing the XD transcript nor does it synergize with the cytokine in this respect. Moreover, maximally active concentrations (Reich & Pfeffer, 1990) of two non-specific inhibitors of protein kinase C, i.e. staurosporine and

Fig. 7. Effect of protein kinase C activators and inhibitors (a) and dibutyryl cyclic AMP (b) on the induction of XD mRNA by IFN- α A/D

Cells were cultured for 18 h in medium alone (control) or in medium containing the indicated compounds. Total RNA was extracted and used for Northern-blot analysis ($20\text{ }\mu\text{g/lane}$). The same filters were sequentially hybridized with XD, oligoAS and α -actin cDNAs. (a) PMA, phorbol 12-myristate 13-acetate (100 nM); Sta, staurosporine (100 nM); H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine ($50\text{ }\mu\text{M}$). (b) cAMP, dibutyryl cyclic AMP (1 mM). The positions of the size markers (28S and 18S rRNA) are indicated.

Fig. 8. Effect of IFN- α A/D on the expression of XD transcript in various cell lines

The indicated cell lines were cultured for 18 h in medium alone (control), or in medium containing IFN- α A/D (10^3 units/ml) (IFN) for 18 h. Total RNA was extracted and used for Northern-blot analysis ($20 \mu\text{g}/\text{lane}$). The same filter was sequentially hybridized with XD, oligoAS and α -actin cDNAs. B16, B16 melanoma cells; F9, F9 teratocarcinoma cells; NIH3T3, NIH3T3 fibroblastic cells; L929, L929 fibroblastic cells. The positions of the size markers (28S and 18S rRNA) are indicated.

H7, do not block the upregulation of XD mRNA by IFN- α A/D. Similar results are observed with the oligoAS transcript. Dibutyl cyclic AMP, an analogue of cyclic AMP capable of permeating through the plasma membrane, affects neither the levels of XD mRNA nor IFN- α A/D induction of the transcript (Fig. 7b). Furthermore, incubation of L929 cells for 18 h with indomethacin or nordihydroguaiaretic acid, which are inhibitors of the cyclooxygenase- and the lipoxygenase-mediated metabolism of arachidonic acid respectively, do not have any effect on the induction of XD mRNA by IFN- α A/D (results not shown).

To investigate whether the induction of XD mRNA by IFN is peculiar to L929 cells and if it is always parallel with the induction of the oligoAS message, experiments were performed on other cell lines. B16 melanoma cells were selected because IFN- α is used in the treatment of melanomas (Gresser, 1991). F9 teratocarcinoma cells were chosen because they contain an adenovirus EIA-like activity (Imperiale *et al.*, 1984) and it is known that adenoviruses counteract some of the effects of IFNs through the action of similar molecules (Ackrill *et al.*, 1991). NIH3T3 cells are of interest because, like L929 cells, they represent a fibroblastic cell line. Fig. 8 demonstrates that the induction of XD transcript is not limited to L929 cells but it is observed also in NIH3T3 cells, even though this cell line shows a basal level of XD

transcript that is higher than that observed in L929 cells and it is less responsive to the induction of the XD transcript by IFN (about 3-fold relative to control conditions). B16 melanoma cells as well as F9 teratocarcinoma cells show no increase in XD transcript after treatment with 10^3 units of IFN- α A/D/ml. This effect is not due to a lack of IFN receptors on their plasma membrane since oligoAS mRNA is increased in both cell lines after treatment with the cytokine.

Despite their usefulness as an experimental model in the study of the regulation of XD transcript by IFNs, L929 cells are devoid of XD and XO activities in both the absence and presence of the cytokine. In fact, experiments performed using a range of IFN- α A/D concentrations from 0.1 to 10^5 units/ml and a time-frame of treatment from 4 to 72 h show XD and XO activities below the limit of detection of the sensitive radiometric enzyme assay used (Reiners *et al.*, 1987). In this respect, the only cells so far tested that show detectable XD and XO enzyme activities are NIH3T3 cells. This cell line contains XD and XO activities of 2.93 ± 0.37 and 0.60 ± 0.07 (mean $\pm$ s.d., $n = 3$) munits/mg of protein respectively under basal conditions and 4.90 ± 0.50 and 1.00 ± 0.12 (mean $\pm$ s.d., $n = 3$) munits/mg of protein respectively after 48 h of treatment with 10^3 units of IFN- α A/D/ml.

DISCUSSION

In the experiments reported in the present paper the expression of XD mRNA in L929 fibroblastic cells after IFN- α A/D treatment was studied in detail at the molecular level. The data presented suggest that the XD gene is directly regulated by IFNs, since the induction of the respective transcript is rather fast and is not blocked by cycloheximide. Moreover, the induction of XD mRNA is, at least in part, the result of an increase in the transcriptional activity of the gene. In fact, the induction is sensitive to actinomycin D, the XD mRNA half-life is not affected by IFN treatment and, more directly, the rate of transcription of nascent mRNAs is increased by the cytokine. The increase in transcriptional activity of the XD gene, as determined by nuclear run-on assays, is, however, lower than expected on the basis of the Northern-blot analysis. This situation has been observed with different stimuli for other genes, which, like the XD gene, are already expressed under basal conditions and have a long half-life (Gianni *et al.*, 1991; Glesne *et al.*, 1991). Early post-transcriptional nuclear events seem to play a major role in the observed induction of the mature transcript, as suggested by the Northern-blot data obtained after extraction of RNA from nuclear fractions. Despite the low levels of increase in the transcriptional rate of the XD gene by IFN- α A/D, the data presented are consistent with the presence of an IFN-stimulated responsive element (ISRE) (Cohen *et al.*, 1988; Rutherford *et al.*, 1988) somewhere within the XD gene. The ISRE would allow the regulation of the XD gene by type-I IFNs as a consequence of its interaction with one of the transcriptional trans-activating factors that are known to be under the control of this family of cytokines (Kerr & Stark, 1991). More specifically, the induction of the transcriptional activity of the XD gene might be due to the activation of the ISGF3 transcriptional complex (E complex according to another nomenclature) (Kerr & Stark, 1991; Levy *et al.*, 1989). ISGF3 is present under basal conditions in the cytoplasm as an inactive complex (Levy *et al.*, 1989). Activation is triggered by as yet unidentified post-translational mechanisms by IFN and it is followed by subsequent export to the nucleus where it binds to the ISRE (Kerr & Stark, 1991). The activation of ISGF3 is complete in a time-frame of minutes and does not require protein synthesis *de novo*, two features that are consistent with the induction of XD gene transcriptional activity by IFN- α A/D in our experimental conditions. However, the induction

of XD mRNA is not influenced by arachidonic acid metabolism, which seems to play a role in the activation of ISGF3, at least in terms of ISRE-binding activity, in other cellular contexts (Hannigan & Williams, 1991). The cloning of the IFN-responsive regulatory elements of the XD gene and their structural characterization will clarify the role of ISGF3 (if any) or other IFN-induced *trans*-acting factors in the induction of the expression of the XD gene.

The biochemical signals that stand between the interaction of type-I IFNs with their receptor and the activation of the transcription of the genes under the control of these cytokines are still unknown.

Protein kinase C has been implicated as a mediator of the growth-suppressive signals of IFNs in haematopoietic cells (Fan *et al.*, 1988; Reich & Pfeffer, 1990; Tiefenbrun & Kimchi, 1991). Arachidonic acid metabolites (Hannigan & Williams, 1991) and the cyclic AMP-dependent protein kinase system (Nagata *et al.*, 1984) have also been implicated as possible second-messenger molecules of IFN-mediated effects. However, our data are against any involvement of protein kinase C, cyclic AMP or arachidonic acid metabolism (via either the lipoxygenase or the cyclooxygenase system) in the induction of XD mRNA in L929 cells. Reactive oxygen intermediates have been proposed as possible second messengers in the activation of the transcriptional factor NF κ B (Schreck *et al.*, 1991). To test a possible role for these oxygen species in the induction of XD mRNA by IFN- α A/D, experiments using various concentrations of *N*-acetylcysteine, a known scavenger of free radicals, were performed. All the experiments produced negative results (not shown).

Since the XD mRNA has been shown to be under the direct control of IFNs *in vivo* (Terao *et al.*, 1992), it is important to compare its behaviour with that of genes that are classically considered as markers for the biological activity of these cytokines. For this reason, the modulation of XD mRNA by IFNs in L929 cells was compared with that of oligoAS. The XD gene behaves similarly to the oligoAS gene as far as its kinetics of induction, dose-response curve and cycloheximide insensitivity are concerned. However, XD requires the continuous presence of IFN- α A/D to sustain its own induction, whereas as little as 10 min of contact between the cells and the cytokine are enough to obtain a maximal and long-lasting accumulation of oligoAS transcript. This difference in behaviour might reflect diversities in the mechanisms of induction of the two genes, which are underscored by the differential expression of XD and oligoAS transcripts in at least two cell lines. There are, in fact, cells, such as F9 teratocarcinoma and B16 melanoma, where the XD gene does not respond to IFN- α A/D but the oligoAS gene does.

Besides its regulation at the level of transcription and stabilization of heterogeneous nuclear RNA, a third mechanism by which the expression of the XD gene may be controlled is represented by translation or activation of the protein. In fact, L929 cells respond to IFN- α A/D and IFN- γ by a dramatic increase in XD mRNA accumulation, which is, however, not accompanied by a parallel increase in XD or XO activity. XD and XO activities are below the level of detection in both control and IFN- α A/D-stimulated conditions. The absence of enzyme activity is not due to poor sensitivity of the assay, since XD and XO activities are easily measured *in vivo* in the mouse liver in conditions where similar levels of XD mRNA accumulation are attained (Terao *et al.*, 1992). Moreover, XD and XO activities do not seem to be dependent on double-stranded RNA, since addition of poly(I/C) in L929 cell extracts before the assay does not stimulate the two enzyme activities (F. Falciani, P. Ghezzi, M. Terao, G. Cazzaniga & E. Garattini, unpublished work). It is yet to be established whether L929 cells express a genetically mutated and inactive form of XD, even though this seems

unlikely, since the enzyme activity is measurable under stimulation with molybdenum oxide (F. Falciani, P. Ghezzi, M. Terao, G. Cazzaniga & E. Garattini, unpublished work). At present, the lack of specific antibodies recognizing mouse XD prevents us determining whether the block in the expression of XD activity is a translational or post-translational event. The only cells (among the limited number of cell lines so far tested) that express measurable XD and XO activity are NIH3T3. These cells demonstrate XD and XO activities that are easily measured in both control and IFN-stimulated conditions. However, this cell line responds poorly to IFN- α A/D treatment and cannot be used to study the molecular mechanisms underlying the induction of XD mRNA by the cytokine. Furthermore, NIH3T3 is not commonly used as a target cell for the biological effects of IFNs. Whatever mechanism is responsible for the lack of expression of XD and XO activities in L929 and other cell lines under basal as well as stimulated conditions, it is clear that this fact rules out a possible involvement of this protein in the antiviral response induced by IFNs in L929 cells. It is yet to be established whether this protein plays any role in the antiproliferative or immunomodulatory activity of IFNs. Further experiments are required to clarify these points.

This work was supported in part by grants from the Consiglio Nazionale delle Ricerche (CNR), progetto finalizzato 'Ingegneria Genetica' contract no. 91.00060.PF99 and the Associazione Italiana per la Ricerca contro il Cancro (AIRC). The financial support received from the Mario Negri Institute Foundation of New York through a contribution of Donald P. and Jeanne Kahn of Miami, FL, U.S.A., is also gratefully acknowledged. F. F. is a recipient of a fellowship from the Fondazione Attilia Pofferi.

REFERENCES

- Ackrill, A. M., Foster, G. R., Laxton, C. D., Flavell, D. M., Stark, G. R. & Kerr, I. M. (1991) *Nucleic Acids Res.* **19**, 4387-4393
- Aguet, M. (1991) *Br. J. Haematol.* **79** Suppl. 1, 6-8
- Benech, P., Merlin, G., Revel, M. & Chebath, J. (1985a) *Nucleic Acids Res.* **13**, 1267-1281
- Benech, P., Mory, Y., Revel, M. & Chebath, J. (1985b) *EMBO J.* **4**, 2249-2256
- Boss, J. M. & Strominger, J. L. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9139-9143
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254
- Carpani, G., Racchi, M., Ghezzi, P., Terao, M. & Garattini, E. (1990) *Arch. Biochem. Biophys.* **279**, 237-241
- Cohen, B., Peretz, D., Vaiman, D., Benech, P. & Chebath, J. (1988) *EMBO J.* **7**, 1411-1419
- Dell Corte, E. & Stirpe, F. (1968) *Biochem. J.* **108**, 349-351
- Della Corte, E. & Stirpe, F. (1972) *Biochem. J.* **126**, 739-745
- Fan, X. D., Goldberg, M. & Bloom, B. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5122-5125
- Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6-13
- Ghezzi, P., Bianchi, M., Mantovani, A., Spreafico, F. & Salmons, M. (1984) *Biochem. Biophys. Res. Commun.* **119**, 144-149
- Ghezzi, P., Bianchi, M., Gianera, L., Landolfo, S. & Salmons, M. (1985) *Cancer Res.* **45**, 3444-3447
- Gianni, M., Studer, M., Carpani, G., Terao, M. & Garattini, E. (1991) *Biochem. J.* **274**, 673-678
- Glesne, D. A., Collart, F. R. & Huberman, E. (1991) *Mol. Cell. Biol.* **11**, 5417-5425
- Greenberg, M. E. & Ziff, E. B. (1984) *Nature (London)* **311**, 433-438
- Gresser, I. (1991) *Br. J. Haematol.* **79** Suppl. 1, 1-5
- Hannigan, G. E. & Williams, B. R. G. (1991) *Science* **251**, 204-207
- Hatch, C. L. & Bonner, W. M. (1988) *Nucleic Acids Res.* **16**, 1113-1124
- Imperiale, M. J., Kao, H. T., Feldman, L. T., Nevins, J. R. & Strickland, S. (1984) *Mol. Cell. Biol.* **4**, 867-874
- Isaac, A. & Lindenmann, J. (1957) *Proc. R. Soc. London (Biol.)* **147**, 258-267
- Kerr, I. M. & Stark, G. R. (1991) *FEBS Lett.* **285**, 194-198
- Kirchner, H. & Shellekens, H. (eds.) (1984) *The Biology of the Interferon System*, Elsevier, Amsterdam

- Lengyel, P. (1982) *Annu. Rev. Biochem.* **51**, 251–282
- Levy, D. E., Kessler, D. S., Pine, R. & Darnell, J. E., Jr. (1989) *Genes Dev.* **3**, 1362–1371
- Luster, A. D. & Ravetch, J. V. (1987) *Mol. Cell. Biol.* **7**, 3723–3731
- Marzluff, W. F. & Huang, R. C. C. (1984) in *Transcription and Translation, A Practical Approach* (Hames, B. D. & Higgins, S. J., eds), pp. 89–129, IRL Press, Oxford
- Minty, A. J., Caravatti, M., Robert, B., Cohen, A., Daubas, P., Weydert, A., Gros, F. & Buckingham, M. E. (1981) *J. Biol. Chem.* **256**, 1008–1014
- Nagata, Y., Rosen, O. M., Makman, N. H. & Bloom, B. R. (1984) *J. Cell Biol.* **98**, 1342–1347
- Pestka, S., Langer, J. A., Zoon, K. C. & Samuel, C. (1987) *Annu. Rev. Biochem.* **56**, 727–777
- Porter, A. C. G., Chernajovsky, Y., Dale, T. C., Gilbert, C. S., Stark, G. R. & Kerr, I. M. (1988) *EMBO J.* **7**, 85–92
- Rambaldi, A., Young, D. C. & Griffin, J. D. (1987) *Blood* **69**, 1409–1413
- Reich, N. C. & Pfeffer, L. M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8761–8765
- Reich, N., Evans, B., Levy, D., Fahey, D., Knight, E., Jr. & Darnell, J. E., Jr. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6394–6398
- Reid, L. E., Brasnett, A. H., Gilbert, C. S., Porter, A. C. G., Gewart, D. R., Stark, G. R. & Kerr, I. M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 840–844
- Reiners, J. J., Jr., Pence, B. C., Barcus, M. C. S. & Cantu, A. R. (1987) *Cancer Res.* **47**, 1775–1779
- Rutherford, M. N., Hannigan, G. E. & Williams, B. R. G. (1988) *EMBO J.* **7**, 751–759
- Schneck, J., Rager-Zisman, B., Rosen, O. M. & Bloom, B. R. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1879–1883
- Schreck, R., Rieber, P. & Baeuerle, P. A. (1991) *EMBO J.* **10**, 2247–2258
- Seiler-Tuyns, P. & Birnstiel, M. L. (1981) *J. Mol. Biol.* **157**, 607–625
- Staeheli, P. (1990) *Adv. Vir. Res.* **38**, 147–200
- Terao, M., Cazzaniga, G., Ghezzi, P., Bianchi, M., Falciani, F., Perani, P. & Garattini, E. (1992) *Biochem. J.* **283**, 863–870
- Tiefenbrun, N. & Kimchi, A. (1991) *Oncogene* **6**, 1001–1007
- Weil, J., Epstein, C. J., Epstein, L. B., Sedmak, J. J., Sabran, J. L. & Grossberg, S. E. (1983) *Nature (London)* **301**, 437–439

Received 6 January 1992; accepted 4 February 1992