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Molecular cloning of a cDNA coding for mouse liver xanthine dehydrogenase

Regulation of its transcript by interferons *in vivo*

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The cDNA coding for xanthine dehydrogenase (XD) is isolated from mouse liver mRNA by cross-hybridization with a DNA fragment of the *Drosophila melanogaster* homologue. Two λ bacteriophage overlapping clones represent the copy of a 4538-nucleotide-residue-long transcript with an open reading frame of 4005 nucleotide residues, coding for a putative polypeptide of 1335 amino acid residues. Comparison of the deduced amino acid sequence of the mouse XD with those of the *Drosophila* and the rat homologues shows a high conservation of this protein (55% identity between mouse and *Drosophila*, and 94% identity between mouse and rat). RNA blotting analysis demonstrates that interferon-α (IFN-α) and its inducers, i.e. poly(I)·poly(C), bacterial lipopolysaccharide (LPS) and tilorone {2,7-bis-[2-(diethylamino)ethoxy]fluoren-9-one}, increase the expression of XD mRNA in liver. Poly(I)·poly(C) also induces XD mRNA in several other tissues *in vivo*. Protein synthesis *de novo* is not required for the elevation of XD mRNA after IFN-α treatment, since cycloheximide does not block the induction. The elevation of XD mRNA concentration is relatively fast and precedes the induction of both XD and xanthine oxidase (XO) enzymic activities.

INTRODUCTION

Xanthine oxidoreductase is the enzyme system that catalyses the oxidation of hypoxanthine to xanthine and subsequently to uric acid (Dixon & Webb, 1964). The enzyme is a single-gene product and it exists in two separate but interconvertible forms, xanthine dehydrogenase (EC 1.1.1.204; XD) and xanthine oxidase (EC 1.1.3.22, formerly EC 1.2.3.2; XO). The former uses NAD^+ as the acceptor of reducing equivalents whereas the latter transfers them to molecular O_2. XD and XO enzymic activities are contemporaneously detected in a variety of tissues, even though the conversion of XD into XO makes the latter the most readily extractable form (Gilbert & Bergel, 1964; Krenitsky *et al.*, 1986; Carpani *et al.*, 1990). In fact, XD is converted into XO in various experimental conditions *in vitro* (Della Corte & Stirpe, 1968, 1972). The conversion of XD into XO induced by oxidation is reversible after treatment with reducing agents, and partial proteolytic cleavage irreversibly transforms XD into XO (Granger *et al.*, 1981; Engerson *et al.*, 1987; Oda *et al.*, 1989). *In vivo*, it is known that XO is produced from XD in various pathological conditions, where it has been proposed to play an important pathogenetic role (Granger *et al.*, 1981; Engerson *et al.*, 1987; Reiners *et al.*, 1987; Elsayed & Tierney, 1989). In particular, this enzyme seems to be primarily responsible for the vascular injury associated with intestinal (Parks & Granger, 1983), cardiac (McCord, 1985; Terada *et al.*, 1991) and hepatic ischaemia (de Groot & Littauer, 1988; Brass *et al.*, 1991). The involvement of XO in tissue damage is probably related to the ability of this enzyme to produce highly reactive $O_2^{\cdot-}$ anions (Fridovich, 1970).

XO seems to mediate some of the toxic effects produced by interferons (IFNs) and their inducers in the mouse (Renton *et al.*, 1984; Ghezzi *et al.*, 1984, 1985; Deloria *et al.*, 1985). In fact, recombinant IFNs as well as bacterial lipopolysaccharide (LPS) and poly(I)·poly(C) induce XO enzymic activity and depress cytochrome *P*-450 concentrations in mouse liver. This toxic effect is prevented by pretreatment of the animals with allopurinol and *N*-acetylcysteine, a specific inhibitor of XO and a scavenger of oxygen radicals respectively (Taylor *et al.*, 1980; Roy & McCord, 1982; Ghezzi *et al.*, 1985; Bindoli *et al.*, 1988).

To study the molecular mechanisms underlying the elevation of XO enzymic activity by IFNs and their inducers, the cDNA coding for XD was isolated from mouse liver mRNA. With the use of this cDNA clone as a probe, the elevation of XO/XD activity by IFN-α and its inducers is demonstrated to be mostly due to an increased accumulation of XD mRNA.

MATERIALS AND METHODS

Animal treatments

CD-1 mice obtained from Charles River Italia (Calco, Como, Italy) weighing approx. 30 g were intraperitoneally treated with poly(I)·poly(C) (Sigma Chemical Co., St. Louis, MO, U.S.A.) (10 mg/kg body wt.), recombinant IFN-α (A/D) (IFN-α; 10^7 units/mg of protein) (Roche, Nutley, NJ, U.S.A.) (1.5×10^5 units/kg body wt.), LPS (Sigma Chemical Co.) (2 mg/kg body wt.), recombinant human interleukin-1β (10^7 units/mg of protein) (Sclavo, Siena, Italy) (30 μg/kg body wt.) or recombinant human tumour necrosis factor-α (10^7 units/mg of protein) (Cetus Corp., Emeryville, CA, U.S.A.) (30 μg/kg body wt.). IFN-α (A/D) is a human hybrid IFN-α type that crosses the species barrier and acts on the IFN-α/β receptor (Rehberg *et al.*, 1982; Jung *et al.*, 1988). In one experiment, cycloheximide (Sigma Chemical Co.) (150 mg/kg)

Abbreviations used: the gene and the transcript coding for xanthine dehydrogenase (XD) and xanthine oxidase (XO) are referred to as the *XD* gene and *XD* transcript throughout the paper; IFN, interferon; LPS, lipopolysaccharide.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X62932.

was administered to the animals 2 h before the treatment with IFN or saline alone to study the effect of inhibition of protein synthesis on the induction of liver XD mRNA by IFN-α itself. Tilorone {2,7-bis-[2-(diethylamino)ethoxy]fluoren-9-one} (Merrel Dow Pharmaceutical, Cincinnati, OH, U.S.A.) (75 mg/kg body wt.) was administered orally. The effect of LPS on the induction of liver XD mRNA was also tested in two different mouse strains, C3H/HeJ (a strain resistant to the effect of LPS) and C3H/HeN (a strain with the same genetic background as C3H/HeJ, but sensitive to LPS) (Charles River) (O'Brien *et al.*, 1982).

Isolation of mouse liver XD cDNAs

In order to obtain molecular probes that could be used for the isolation of mouse XD cDNA, two stretches of the *Drosophila melanogaster XD* gene (Keith *et al.*, 1987; Lee *et al.*, 1987), between nucleotide residues 1496–2018 and 3014–3643 respectively, were amplified according to the instructions of the manufacturer (Cetus Perkin–Elmer, Norwalk, CT, U.S.A.). Amplified fragments were subcloned in pBluescript SK (Stratagene) with standard techniques (Maniatis *et al.*, 1982) and their identity was verified by seqencing.

The amplified oligo(dT)-primed mouse liver cDNA library in λgt10 was purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). The oligo(dT) and the randomly primed cDNA libraries inserted in the *Eco*RI site of λZAP II vector (Stratagene) were constructed with the use of liver polyadenylated RNA obtained from mice treated with 10 mg of poly(I)·poly(C)/kg body wt. according to standard protocols (Huynh *et al.*, 1985). In all, 7×10^5 plaques from the commercial cDNA library were screened with the ^{32}P-labelled *Drosophila XD* gene probes. The filters were hybridized at 53 °C in a solution containing 5 × SSC, 5 × Denhardt's and 100 μg of salmon sperm DNA/ml (Maniatis *et al.*, 1982) for 20 h. (1 × SSC is 0.15 M-NaCl/15 mM-sodium citrate buffer, pH 7; 1 × Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% BSA.) The filters were subsequently washed in 2 × SSC/0.1% SDS at room temperature twice for 20 min, and in 0.5 × SSC/0.1% SDS at 53 °C twice for 20 min. One positive clone hybridizing only with the most 5′ *Drosophila XD* gene fragment was isolated and the insert was subcloned in the *Eco*RI site of pBluescript SK (XDgt1).

To obtain the 3′-end of mouse XD cDNA, 50000 primary recombinant plaques derived from the oligo(dT)-primed poly(I)·poly(C)-induced liver cDNA library constructed in λzap II vector were screened with ^{32}P-labelled XDgt1 insert as a probe, in standard conditions (Maniatis *et al.*, 1982). Positive clones were isolated and the DNA inserts were recovered as subclones in pBluescript KS according to the instructions of the manufacturer (Stratagene). Only one clone (XDzap12), containing the longer insert, was further characterized.

To obtain part of the 5′-end of XD cDNA, two anti-sense strand oligonucleotides (positions 1630–1654 and 1594–1623 in Fig. 2) were synthesized. The former was used to prepare a cDNA library in the *Eco*RI site of the plasmid pUC18 (Pharmacia, Uppsala, Sweden) by primer extension of polyadenylated RNA obtained from the liver of poly(I)·poly(C)-treated mice (Huynh *et al.*, 1985). Approx. 2000 bacterial colonies were screened with the latter oligonucleotide under the conditions described by Wood *et al.* (1985). Four positive clones (XDpuca1–4) were isolated and sequenced.

The rest of the 5′ portion of the XD cDNA was isolated from a λZAP II randomly primed liver cDNA library (80000 primary recombinant plaques) with the use of ^{32}P-labelled XDpuca2 as a probe. Seventy positive clones were isolated, and the one containing the longest insert was recovered in pBluescript KS and further characterized (XDzap64).

Sequence analysis

Nucleotide sequence on double-stranded DNA templates was performed according to the dideoxy chain-termination method (Sanger *et al.*, 1977) with the use of Sequenase (United States Biochemical Corporation, Cleveland, OH, U.S.A.) or T7 DNA polymerase (Pharmacia) according to the instructions of the manufacturers. The sequences of XDzap64, XDzap12 and the respective subclones derived from them were determined on both strands. The primers used for the sequencing reactions were either vector primers or specific oligonucleotides (17–22 nucleotide residues long) synthesized based on the information obtained from former sequence analysis. Oligonucleotides were synthesized on a Beckman Sys-200 oligonucleotide synthesizer (Beckman Instruments, Palo Alto, CA). Computer analysis of the DNA sequences was performed with the Microgenie sequence analysis system (Beckman) and the McMolly Soft Gene Berlin software (Apple Computer, Cupertino, CA, U.S.A.).

RNA blotting analysis

Total RNA and polyadenylated RNA were prepared from various tissues according to standard procedures (Maniatis *et al.*, 1982).

For RNA blotting analysis, total RNA (20 μg) or polyadenylated RNA (5 μg) was fractionated on a 1.2% agarose gel containing 6% formaldehyde and blotted onto synthetic nylon membranes (Gene Screen Plus; New England Nuclear, Boston, MA, U.S.A.). These membranes were hybridized to the 1.8 kb *Eco*RI fragment of XDgt1, or to the mouse actin cDNA (Minty *et al.*, 1981). The probes were labelled to a specific radioactivity of 1×10^9–2×10^9 c.p.m./μg by using hexanucleotide primers and [^{32}P]dCTP (Feinberg & Vogelstein, 1983). Hybridization was performed at 60 °C for 20 h in a solution containing 1 M-NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate (Sigma Chemical Co.), 100 μg of salmon sperm DNA (Boehringer)/ml and 1×10^6–2×10^6 c.p.m./ml of ^{32}P-labelled probe. The membranes were washed twice with 2 × SSC/1% SDS for 30 min at 65 °C and then with 0.1 × SSC for 30 min at room temperature. The membranes were dried and exposed to Kodak X-Omat X-ray films (Eastman Kodak Co., Rochester, NY, U.S.A.) with two intensifying screens (Dupont Cronex; Dupont de Nemours and Co., Wilmington, DE, U.S.A.) at −70 °C.

XD/XO assay

At 12 h after intraperitoneal administration of poly(I)·poly(C) or saline as a control, animals were killed by cervical dislocation. Liver was excised, washed in saline, blotted dry and homogenized in 10 vol. of 100 mM potassium phosphate buffer, pH 7.8, containing 1 mM-EDTA and 3 mM-phenylmethanesulphonyl fluoride. Solid $(NH_4)_2SO_4$ was added to the homogenate to a final concentration of 20% (w/v), and the mixture was centrifuged at 12000 *g*. XO activity in the supernatant was recovered by addition of solid $(NH_4)_2SO_4$ to a final concentration of 40% (w/v). After centrifugation, the pellet was resuspended in 1 ml of 100 mM-potassium phosphate buffer, pH 7.8, containing 1 mM-EDTA and 1 mM-phenylmethanesulphonyl fluoride and dialysed overnight against 100 vol. of the same buffer.

The enzyme assay was performed in a Beckman DU-65 spectrophotometer at 25 °C (Stirpe & Della Corte, 1969). For the measurement of XD + XO activities, the assay mixture contained 100 mM-sodium pyrophosphate buffer, pH 8.5, 0.2 mM-EDTA, 0.1 mM-xanthine, 0.4 mM-NAD^+ and 50 μl of enzyme sample in a final volume of 1 ml. XO activity was determined in the absence of NAD^+, in the same incubation mixture as above. XD was determined by subtracting XO from XD + XO. The enzyme assays were performed in conditions of linearity in respect to

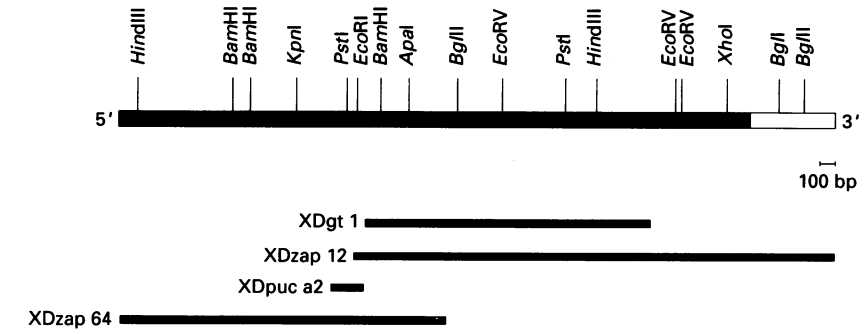

Fig. 1. Structural organization and physical map of mouse liver XD cDNA

The thick line represents the physical map of mouse liver XD cDNA from its 5'-end to the 3'-end (left to right). Sites for relevant restriction endonucleases are indicated. The black box and the white box indicate the protein coding region and the 3' untranslated region respectively. Thin lines shown at the bottom of the Figure represent inserts of recombinant λ phages (XDgt1, XDzap12, XDzap64) or recombinant plasmid (XDpuca2). The clones XDzap64 and XDzap12 were sequenced completely in both directions, whereas XDgt1 and XDpuca2 were sequenced only in one direction.

protein and time. One unit of enzyme activity is defined as 1 nmol of uric acid produced/min (Suleiman & Stevens, 1985). The data were normalized for the content of proteins determined by the Bio-Rad kit (Bio-Rad Laboratories, Richmond, CA) with BSA as a standard (Sigma Chemical Co.) (Bradford, 1976).

RESULTS AND DISCUSSION

Cloning and structure of the cDNA coding for mouse liver XD

When the molecular cloning of the cDNA coding for mouse XD was started, no data on the primary structure of this enzyme in higher eukaryotes were available, except for that of *Drosophila melanogaster*, which was identified and isolated by genetic complementation of the Rosy locus (Bender *et al.*, 1983; Keith *et al.*, 1987; Lee *et al.*, 1987). XDgt1 was thus isolated by cross-hybridization with the *Drosophila melanogaster XD* gene. Isolation of the other overlapping clones was as detailed in the Materials and methods section. The restriction map of the mouse XD cDNA as defined by the isolated overlapping clones is shown in Fig. 1.

The total length of the mouse XD cDNA is 4538 nucleotide residues (Fig. 2). The longest open reading frame deduced from the DNA sequence is 4005 nucleotide residues long, coding for a putative polypeptide of 1335 amino acid residues. This is consistent with the fact that the protein product of this gene has an approximate molecular mass of 150 kDa (Carpani *et al.*, 1990). The assignment of the first ATG codon is presumptive, owing to the lack of *N*-terminal sequence data of the protein. The contention, however, is supported by the protein and cDNA sequences of the rat (Amaya *et al.*, 1990) and the *Drosophila* (Keith *et al.*, 1987; Lee *et al.*, 1987) XD. In these two animal species, the first methionine codon appears at a position similar to that of the mouse cDNA, and the three genes share great similarity in this region of the protein, as described below. The open reading frame of the mouse XD cDNA is followed by a 531-nucleotide-residue-long 3' untranslated region containing a polyadenylation signal, AATAAA (nucleotide residues 4518–4523 in Fig. 2) (Wickens & Stephenson, 1984; Birnstiel *et al.*, 1985) that is located 10 nucleotide residues upstream of the poly(A) tail. The *XD* transcript is devoid of a nucleotide sequence coding for a hydrophobic signal peptide. This feature is consistent with the cytoplasmic localization of the XD protein.

Comparison of mouse XD protein with its rat and *Drosophila* homologues

DNA sequence analysis of the mouse XD cDNA in its coding region demonstrates a high level of similarity with the *Drosophila* (57%) (Keith *et al.*, 1987) and the rat homologue (91%) (Amaya *et al.*, 1990), resulting in a high degree of similarity in their protein products.

The comparison between the deduced primary structure of mouse XD protein and its rat and *Drosophila* homologues is presented in Fig. 3. The overall similarity between mouse and *Drosophila* XD is 55%, whereas it is 94% between the mouse and the rat proteins. The similarity is even higher in their secondary structure, since the hydrophobicity plot is almost superimposable throughout the three animal species (results not shown). The mouse XD polypeptide is composed of 1335 amino acid residues and it has the same length as the *Drosophila* XD, but it is 16 amino acid residues longer than the rat counterpart. A stretch of the mouse sequence coding for 12 amino acid residues (amino acid residues 485–496 in Fig. 3) is found both in mouse and in *Drosophila* XD, but not in rat XD. This could represent a *bona fide* difference relative to the rat, especially in consideration of the fact that it was found in at least two independent clones (XDzap64 and XDpuca2). However, it is also possible that more than one mature XD mRNA transcripts are present both in the rat and in the mouse. A striking similarity among the three XD proteins is found at their *N*-terminal and *C*-terminal regions. The *N*-terminal sequence of the mouse XD is, however, 3 amino acid residues longer than the rat homologue, owing to the presence of a Thr-Arg-Thr tripeptide after the putative translation initiation codon for methionine. One serine residue at position 1291 of the mouse and *Drosophila* XD sequence is lacking in the rat protein.

There are 37 cysteine residues in the mouse XD protein. The positions of all the cysteine residues are perfectly conserved between mouse and rat except for one cysteine residue at position 489, which is located within the stretch of 12 amino acid residues (amino acid residues 485–496) missing in the rat XD. On the other hand, the positions of only 19 cysteine residues of the mouse protein are common to the *Drosophila* counterpart. There is a highly conserved cysteine-rich region at the *N*-terminal portion of the three XD homologues, where 12 out of 14 cysteine residues are common to the three proteins. Some of these cysteine residues might serve as the 2Fe/2S oxidation centres, as already suggested by Amaya *et al.* (1990).

The putative NAD^+-binding site of the mouse XD (amino acid residues 390–403) is very similar to the corresponding site in the rat sequence (Nishino & Nishino, 1989; Amaya *et al.*, 1990), showing 12 out of 14 identical residues. This sequence, however, is not conserved relative to the *Drosophila* counterpart. The

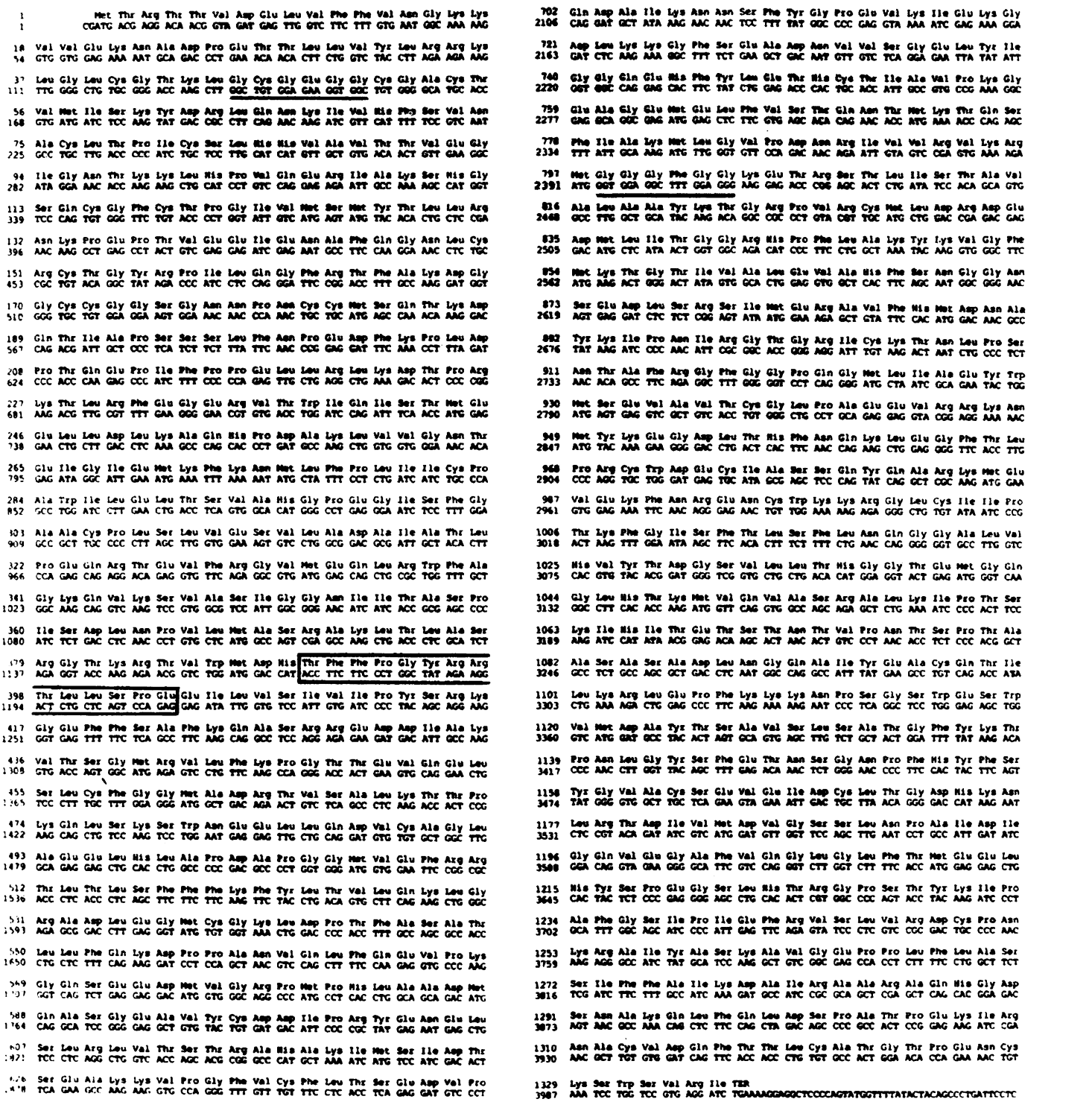

Fig. 2. Nucleotide sequence and deduced amino acid sequence of mouse liver XD

The nucleotide sequence of mouse XD cDNA (lower line) was obtained from the two overlapped cDNA clones XDzap64 (nucleotide residues 1–2000) and XDzap12 (nucleotide residues 1524–4538), and is presented with its deduced amino acid sequence (upper line). Amino acid residues are numbered from the *N*-terminus to the *C*-terminus starting from the putative first methionine residue, whereas nucleotide residues are numbered in the 5′ to 3′ direction. A putative NAD^+-binding site (Nishino & Nishino, 1989; Amaya *et al.*, 1990) is boxed, and two consensus sequences coding for Gly-Xaa-Gly-Xaa-Xaa-Gly, essential for NAD^+ and FAD binding (Guest & Rice, 1984; Williams *et al.*, 1984; Wierenga *et al.*, 1985), are underlined. The polyadenylation signal, AATAAA (Wickens & Stephenson, 1984; Birnstiel *et al.*, 1985), is doubly underlined.

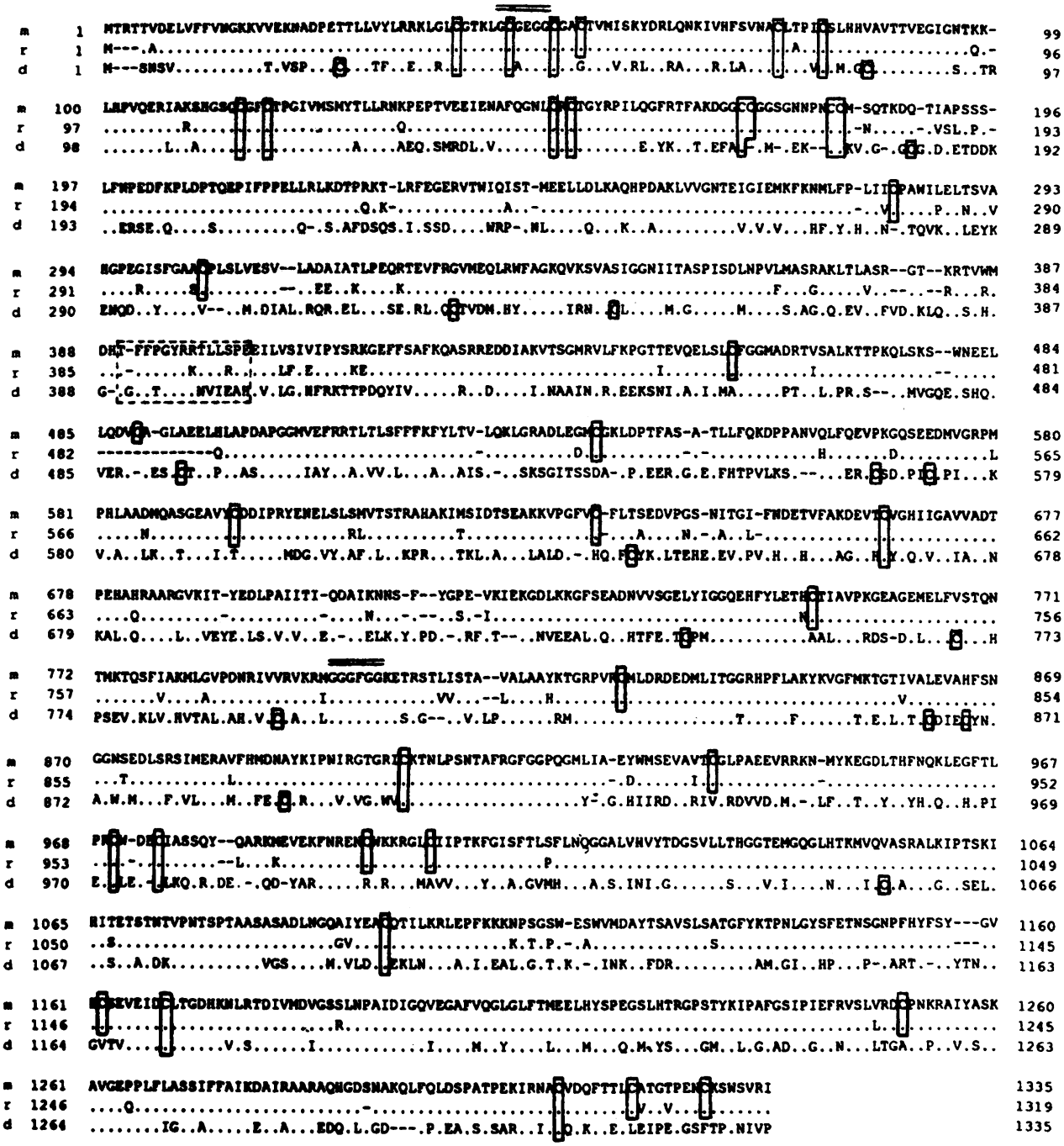

Fig. 3. Amino acid sequence comparison with rat and *Drosophila melanogaster* XD

The deduced amino acid sequences of mouse (m), rat (r) and *Drosophila melanogaster* (d) XD are aligned. The rat sequence is derived from Amaya *et al.* (1990) and the *Drosophila melanogaster* sequence is from Keith *et al.* (1987) and Lee *et al.* (1987). The amino acid residues are indicated by the one-letter code. Numbering starts from the first methionine residue. Identical amino acid residues in the rat and *Drosophila* sequences are indicated as dots except for the first methionine residue, and gaps introduced in the sequence to obtain the best alignment are indicated as bars. Cysteine residues are boxed. Consensus sequences, Gly-Xaa-Gly-Xaa-Xaa-Gly (where Xaa stands for any amino acid residue) for the binding sites of nucleotide cofactors, NAD^+ or FAD, are doubly overlined. The region corresponding to the binding site of NAD^+, as determined by comparison of the 5′-*p*-fluorosulphonylbenzoyladenosine-labelled peptide sequence of chicken XD (Nishino & Nishino, 1989), is boxed by a dotted line.

difference in the environment at the NAD^+-binding site may reflect the fact that, unlike in mammals, XD cannot be converted into XO in this insect. Two consensus amino acid sequences (Gly-Xaa-Gly-Xaa-Xaa-Gly; positions 45–50 and 798–803 in Figs. 2 and 3) that provide the conformation essential for NAD^+ or FAD binding (Guest & Rice, 1984; Williams *et al.*, 1984; Wierenga *et al.*, 1985) are well conserved in the three animal species, suggesting their functional relevance.

Expression of mouse XD gene *in vivo*

The expression of XD gene *in vivo* was studied by RNA blotting analysis with XDgt1 as a probe. Polyadenylated RNA

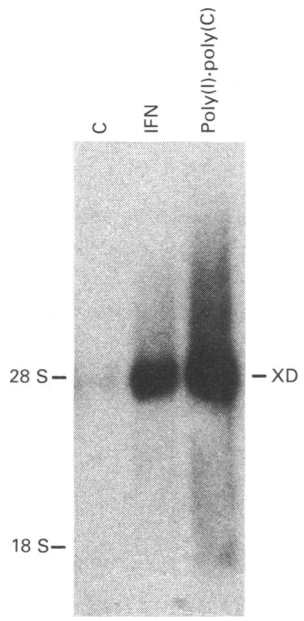

Fig. 4. Induction of mouse liver XD mRNA by IFN-α and poly(I)·poly(C)

Polyadenylated RNA (5 μg/each lane) was extracted from the liver of two mice treated with saline (C), 1.5×10^5 units of IFN-α (IFN)/kg body wt. or 10 mg of poly(I)·poly(C)/kg body wt. The RNA samples were electrophoresed on an agarose/formaldehyde gel and transferred to a nylon membrane for RNA blotting analysis. The positions of the size markers (28 S and 18 S rRNAs) are indicated.

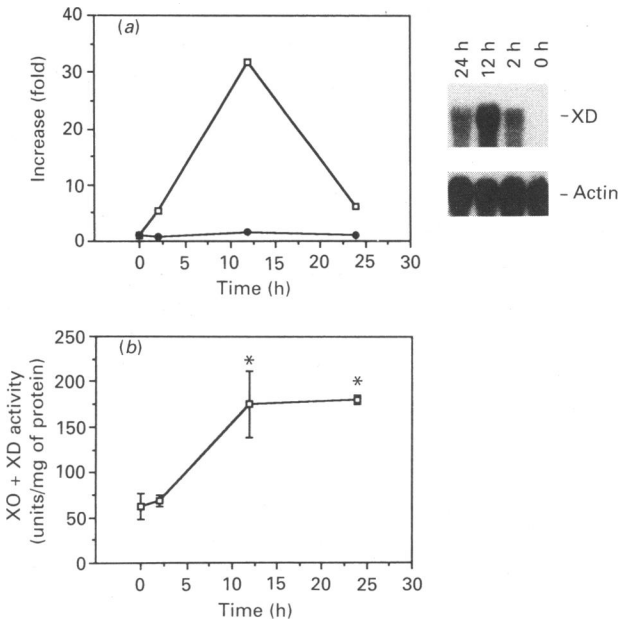

Fig. 5. Time course of the induction of XD mRNA and XO + XD activity in the liver of mice treated with poly(I)·poly(C)

(*a*) Total RNA (20 μg per lane) was extracted from two mice treated with poly(I)·poly(C) (10 mg/kg body wt.) for the indicated amount of time, and used for RNA blotting analysis. The same filter was hybridized sequentially with XD and actin cDNAs. □, XD mRNA; ●, actin mRNA. The quantitative representation of the results shown on the left was obtained after longer exposure of the autoradiogram shown on the right, so as to make the XD band at 0 h detectable. (*b*) The time course of the induction of total XO + XD enzyme activities in the mouse liver is presented. XO + XD activity was measured in tissue extracts obtained from animals treated with poly(I)·poly(C) (10 mg/kg body wt.) for the indicated amount of time. Data are presented as means ± S.E.M. of the values obtained with three separate animals. *Statistically significant relative to the value at time zero ($P < 0.01$; Dunnett's test).

was extracted from mouse liver before and after treatment with IFN-α or poly(I)·poly(C). As shown in Fig. 4, the *XD* transcript is barely detectable before the treatment, whereas after induction with either IFN-α or poly(I)·poly(C) a strong signal, which migrates slightly faster than the 28 S rRNA, is observed.

The kinetics of induction of XD mRNA in the mouse liver after treatment with poly(I)·poly(C) is relatively fast, since the accumulation of the transcript is already visible at 2 h and it attains its maximum level at 12 h (Fig. 5*a*). At 24 h, the level of XD mRNA decreases towards the basal level. To study whether XD mRNA correlates with its translation into catalitically active XO and XD, the sum of the two enzyme activities (XO + XD) was measured. The induction of XD mRNA precedes the elevation of XO + XD enzyme activities (at 2 h, XO + XD activity is not significantly different from the basal level; Fig. 5*b*). At 24 h, XD mRNA decreases dramatically whereas XO + XD activities are still at their plateau. At 48 h, XO + XD activities decrease to the same levels observed at 2 h (results not shown). Considering that XO and XD have very similar K_m and $V_{max.}$ values for xanthine (Bindoli *et al.*, 1985) and that the XD/XO ratio is not significantly changed after administration of poly(I)·poly(C) (for instance, the mean ± S.E.M. is 1.2 ± 0.1 in control conditions compared with 1.1 ± 0.2 after 24 h of poly(I)·poly(C) treatment, confirming the results reported by Ghezzi *et al.*, 1984), the reported increase in XO activity after treatment with poly(I)·poly(C) (Ghezzi *et al.*, 1984, 1985) is thus primarily the result of a dramatic increase in the steady-state concentrations of *XD* transcript. However, a remarkable difference between the maximal induction level of *XD* transcript (about 30-fold) and that of XO + XD enzyme activities (3–10-fold, according to the experiment) is always observed. It is thus possible that not all the transcribed XD mRNA is translated into protein.

To investigate whether other known inducers of IFN-α are also effective in inducing XD mRNA, bacterial LPS and tilorone were administered *in vivo*, and the elevation of the *XD* transcript was compared with that obtained by IFN-α and by poly(I)·poly(C). As shown in Fig. 6(*a*) poly(I)·poly(C), LPS and tilorone are all capable of inducing *XD* gene expression, albeit with different potencies. Furthermore, cycloheximide (a known inhibitor of protein synthesis) does not affect the concentration of *XD* transcript induced by IFN-α, demonstrating that protein synthesis *de novo* is not required for this induction.

Since LPS is known to stimulate many other cytokines besides IFN-α *in vivo*, the specificity of the elevation of XD message by IFN-α was examined. Animals were treated with two cytokines known to be induced by LPS *in vivo*, i.e. tumour necrosis factor and interleukin-1. Fig. 6(*b*) demonstrates that neither interleukin-1 nor tumour necrosis factor is capable of inducing XD mRNA in conditions where poly(I)·poly(C) is effective. Furthermore, the induction by LPS is effective only in the LPS-sensitive mouse strain (C3H/HeN), whereas poly(I)·poly(C) induces XD mRNA accumulation regardless of the sensitivity of the mouse strain to LPS (Fig. 6*c*).

The transcriptional activation of the *XD* gene by poly(I)·poly(C) was tested in various tissues. As shown in Fig. 7, in basal conditions the concentration of XD mRNA is very low in all the tissues studied (the XD mRNA band is visible in liver and intestine only after longer exposures of the autoradiograms). The induction of *XD* transcript is observed not only in liver but in all other tissues tested so far. Except for spleen and brain, high

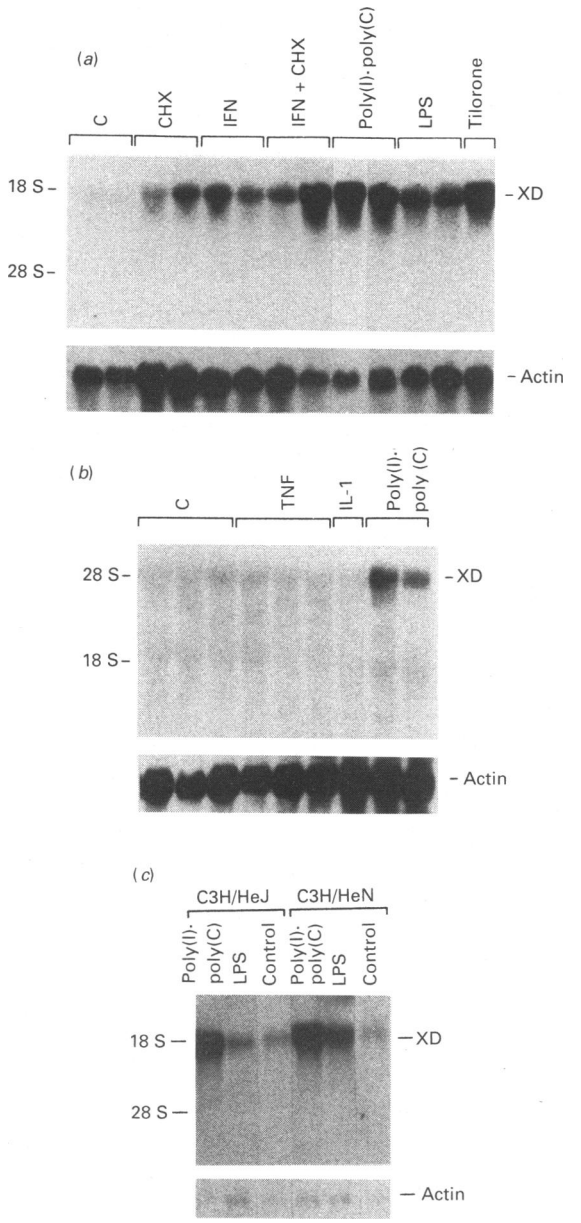

Fig. 6. Effect of cytokines and IFN inducers *in vivo* on the XD mRNA in the mouse liver

(*a*) The induction of XD mRNA by IFN and its inducers was tested after 12 h of treatment *in vivo*. Total RNA (20 μg per lane) was extracted from livers of two mice treated with saline (C), IFN-α (IFN, 1.5×10^5 units/kg body wt.), poly(I)·poly(C) (10 mg/kg body wt.), LPS (2 mg/kg body wt.) or Tilorone (75 mg/kg body wt.) as indicated. In the case of IFN-α, the effect of cycloheximide pretreatment (CHX, 150 mg/kg body wt. administered 30 min before IFN-α or saline) on XD mRNA induction was also tested. Each lane represents the result from a single animal. Filters were sequentially hybridized to XD and actin cDNAs. (*b*) The effects of tumour necrosis factor (TNF, 30 μg/kg body wt.) and interleukin-1 (IL-1, 30 μg/kg body wt.) on XD mRNA concentrations were tested after 12 h of treatment *in vivo*. Poly(I)·poly(C) (10 mg/kg body wt.) was included as a positive control for XD mRNA induction. Total RNA (20 μg per lane) was extracted from mouse liver of control (C) or treated animals as indicated. The filter was sequentially hybridized to XD and actin cDNAs. Each lane represents the result obtained from a single animal. (*c*) The effect of LPS was tested in endotoxin-sensitive (C3H/HeN) and -insensitive (C3H/HeJ) mice. Poly(I)·poly(C) was included as a positive control for XD mRNA induction. Total RNA was extracted 12 h after the treatment of the animals with saline (Control), LPS (2 mg/kg body wt.) or poly(I)·poly(C) (10 mg/kg body wt.). The filters were sequentially hybridized with XD and actin cDNAs.

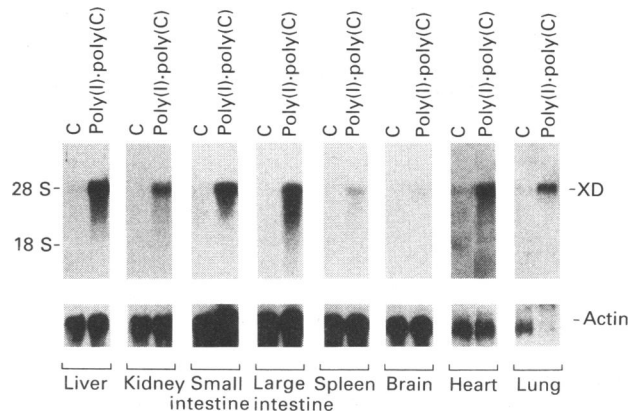

Fig. 7. Induction of XD mRNA by poly(I)·poly(C) in various mouse tissues

Total RNA (20 μg per each lane) was extracted from the indicated tissues 12 h after the treatment with poly(I)·poly(C) (10 mg/kg body wt.) and used for RNA blotting analysis. The filter was sequentially hybridized with XD and actin cDNAs.

levels of accumulation of the XD mRNA after poly(I)·poly(C) treatment are detected in all the other tissues.

This specific induction places *XD* among a few genes whose products have a known enzymic and functional activity that are responsive to IFN treatment *in vivo* (Staheli, 1990). The kinetics of induction as well as the fact that protein synthesis *de novo* is not required for the induction is consistent with the hypothesis that this gene is a primary target of the programmed response of the mammalian cells to IFNs. It is yet to be established, however, whether increased *XD* gene expression is the result of a transcriptional phenomenon and whether the gene itself contains the consensus sequence known to be generally present in the genes that are responsive to treatment with IFNs (Levy *et al.*, 1988). The results presented in this study were obtained with type 1 recombinant IFN-α, but induction of XD mRNA is also achieved by type II IFN (M. Terao, G. Cazzaniga, P. Ghezzi, M. Bianchi, F. Falciani, P. Perani & E. Garattini, unpublished work). Since type I and type II IFNs act through different receptors, it would be interesting to know whether induction of XD mRNA by the two types of IFNs requires the same or different intracellular signals.

The induction of *XD* transcript is not limited to the liver but is observed in all the tissues so far tested, suggesting that the induction of XD mRNA is independent of the cell context on which IFN is acting. However, the cell population(s) involved in XD mRNA induction in the various tissues is still unknown.

As to the role of the XD/XO system in the pathophysiological events triggered by treatments of the animals with IFNs *in vivo*, the involvement of XO in some of the toxic effects mediated by this cytokine are suggested by a series of reports (Ghezzi *et al.*, 1984, 1985; Mannering *et al.*, 1988). IFN-α depresses cytochrome *P*-450 and induces XO in the liver. The depression of cytochrome *P*-450 is inhibited by pretreatment of the animals with allopurinol (a specific inhibitor of XO and XD) and by oxygen-radical scavengers such as *N*-acetylcysteine (Ghezzi *et al.*, 1985). These data suggest XO and $O_2^{\cdot-}$ anions derived from its activity as the mediators of this toxic effect. A key question is represented by the role of the XD/XO system in other biological effects produced by IFNs. It is possible that the increased concentration of XD mRNA is a secondary and incidental event resulting from an alteration in the intracellular purine pool, caused by IFNs. However, it is also possible that the increased production of superoxides derived from the XO system might be related to the antiproliferative or antiviral activity of IFNs. Cloning of the

mouse XD cDNA along with the development of a suitable cell-culture system should be extremely helpful in answering these questions.

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