

# 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  $\lambda$  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- $\alpha$  (IFN- $\alpha$ ) 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- $\alpha$  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.

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## 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<sup>+</sup> as the acceptor of reducing equivalents whereas the latter transfers them to molecular O<sub>2</sub>. 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<sub>2</sub><sup>-</sup> 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- $\alpha$  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- $\alpha$  (A/D) (IFN- $\alpha$ ; 10<sup>7</sup> units/mg of protein) (Roche, Nutley, NJ, U.S.A.) (1.5 × 10<sup>5</sup> units/kg body wt.), LPS (Sigma Chemical Co.) (2 mg/kg body wt.), recombinant human interleukin-1 $\beta$  (10<sup>7</sup> units/mg of protein) (Sclavo, Siena, Italy) (30  $\mu$ g/kg body wt.) or recombinant human tumour necrosis factor- $\alpha$  (10<sup>7</sup> units/mg of protein) (Cetus Corp., Emeryville, CA, U.S.A.) (30  $\mu$ g/kg body wt.). IFN- $\alpha$  (A/D) is a human hybrid IFN- $\alpha$ / $\beta$  type that crosses the species barrier and acts on the IFN- $\alpha$ / $\beta$  receptor (Rehberg *et al.*, 1982; Jung *et al.*, 1988). In one experiment, cycloheximide (Sigma Chemical Co.) (150 mg/kg

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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- $\alpha$  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 sequencing.

The amplified oligo(dT)-primed mouse liver cDNA library in  $\lambda$ gt10 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  $\lambda$ ZAP 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,  $7 \times 10^5$  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  $\times$  SSC, 5  $\times$  Denhardt's and 100  $\mu$ g of salmon sperm DNA/ml (Maniatis *et al.*, 1982) for 20 h. (1  $\times$  SSC is 0.15 M-NaCl/15 mM-sodium citrate buffer, pH 7; 1  $\times$  Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% BSA.) The filters were subsequently washed in 2  $\times$  SSC/0.1% SDS at room temperature twice for 20 min, and in 0.5  $\times$  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  $\lambda$ zap 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  $\lambda$ ZAP 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

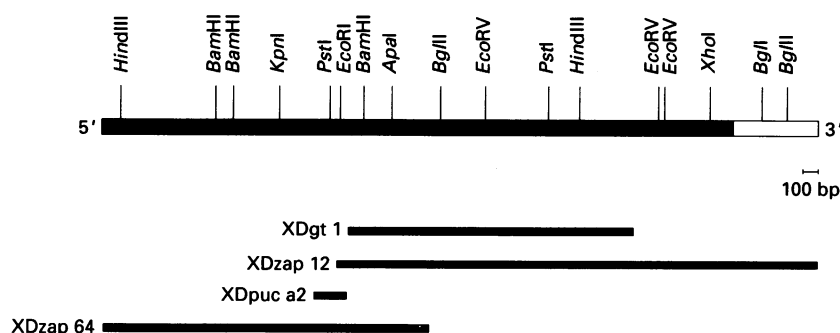
Total RNA and polyadenylated RNA were prepared from various tissues according to standard procedures (Maniatis *et al.*, 1982).

For RNA blotting analysis, total RNA (20  $\mu$ g) or polyadenylated RNA (5  $\mu$ g) 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  $1 \times 10^9$ – $2 \times 10^9$  c.p.m./ $\mu$ g 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  $\mu$ g of salmon sperm DNA (Boehringer)/ml and  $1 \times 10^6$ – $2 \times 10^6$  c.p.m./ml of  $^{32}$ P-labelled probe. The membranes were washed twice with 2  $\times$  SSC/1% SDS for 30 min at 65 °C and then with 0.1  $\times$  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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 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<sup>+</sup> and 50  $\mu$ l of enzyme sample in a final volume of 1 ml. XO activity was determined in the absence of NAD<sup>+</sup>, 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  $\lambda$  phages (XDgt1, XDzap12, XDzap64) or recombinant plasmid (XDpuc a2). The clones XDzap64 and XDzap12 were sequenced completely in both directions, whereas XDgt1 and XDpuc a2 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 XDpuc a2). 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<sup>+</sup>-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

|      |   |      |   |
|------|---|------|---|
| 1    | Met Thr Arg Thr Thr Val Asp Glu Leu Val Phe Phe Val Asn Gly Lys Lys             | 702  | Gln Asp Ala Ile Lys Asn Asn Ser Phe Tyr Gly Pro Gln Val Lys Ile Gln Lys Gly         |
| 1    | CAATG ACG ACG ACA ACG GTA GAT GAG TTG GTC TTC TTT GTG AAT GGC AAA AAG           | 2106 | CAG GAT GCT ATA AAG AAC AAC TCC TTT TAT GAC CCC GAG GTA AAA ATC GAG AAA GGA         |
| 14   | Val Val Glu Lys Asn Ala Asp Pro Glu Thr Thr Leu Leu Val Tyr Leu Arg Arg Lys     | 721  | Asp Leu Lys Lys Gly Phe Ser Glu Ala Asp Asn Val Ser Gly Gln Leu Tyr Ile             |
| 54   | GTG GTG GAG AAA AAT GCA GAC CCT GAA ACA ACA CTT CTG GTC TAT CTT AGA AGA AAG     | 2163 | GAT CTC AAG AAA GGC TTT TCT GAA GCT GAC AAT GTT GTC TCA GGA GAA TTA TAT AAT         |
| 37   | Lys Gly Leu Cys Gly Thr Lys Leu Gly Cys Gly Glu Gly Gly Cys Ala Cys Thr         | 740  | Gly Gly Gln Gln His Phe Tyr Leu Gln Thr His Cys Thr Ile Ala Cys Thr                 |
| 111  | TTG GGG CTG TCC GGC ACC AAG CTT GGC TGT GGA GAA GGT GGC TGT GGG GCA TGC ACC     | 2220 | GCT GGC GAC GAG CAG CAC TTC TAT CTG GAG ACC CAC TCC ACC ATT GGC GTG CCG AAA GGC     |
| 56   | Val Met Ile Ser Lys Tyr Asp Arg Leu Gln Asn Lys Ile Val His Phe Ser Val Asn     | 759  | Glu Ala Gly Glu Met Glu Leu Phe Val Ser Thr Gln Asn Thr Met Lys Thr Gln Ser         |
| 168  | GTG ATG ATC TCC AAG TAT GAC GGC CTT CAG AAC AAG ATC GTT CAT TTT TCC GTC AAT     | 2277 | GAG GCA GGC GAG ATG GAG CTC TTC GTG ACC ACA CAG AAC ACC ATG AAA ACC CAG ACC         |
| 75   | Ala Cys Leu Thr Pro Ile Cys Ser Leu His His Val Ala Val Thr Thr Val Glu Gly     | 778  | Phe Ile Ala Lys Met Leu Gly Val Pro Asp Asn Arg Ile Val Val Arg Val Lys Arg         |
| 225  | GCC TGC TTG ACC CCC ATC TGC TCC TFG CAT CAT GTT GCT ACA ACT GTT GAA GGC         | 2334 | TTT ATT GCA AAG ATG TTG GGT GTT CCA GAC AAC AGA ATT GTA GTC CGA GTG AAA AGA         |
| 94   | Ile Gly Asn Thr Lys Lys Leu His Pro Val Gln Glu Arg Ile Ala Lys Ser His Gly     | 797  | Met Gly Gly Gly Phe Gly Gly Lys Glu Thr Arg Ser Thr Thr Ile Ser Thr Ala Val         |
| 282  | ATA GGA AAC ACC AAG AAG CTG CAT CCT CCT CAG GAG AGA ATT GCC AAA ACC CAT GGT     | 2391 | ATG GGT GCA GGC TTT GGA GGG AAG GAG ACC CCG ACC ACT CTG ATA TCC ACA GCA GTC         |
| 113  | Ser Gln Cys Gly Phe Cys Thr Pro Gly Ile Val Met Ser Met Tyr Thr Leu Leu Arg     | 816  | Ala Leu Ala Ala Tyr Lys Thr Gly Arg Pro Val Arg Cys Met Leu Asp Arg Asp Glu         |
| 339  | TCC CAG TGT GGG TTC TGT ACC CCT GGT AET GTC AFG AGT ATG TAC ACA CTG CTC CGA     | 2448 | GCC TTG GCT GCA TAC AAG ACA GGC CCC CCA GAT CBT TCC ATG CTG GAC CGA GAC PHE         |
| 136  | Asn Lys Pro Glu Pro Thr Val Glu Glu Ile Glu Asn Ala Phe Gln Gly Asn Leu Cys     | 835  | Asp Met Leu Ile Thr Gly Gly Arg His Pro Phe Leu Ala Lys Thr Lys Val Gly Phe         |
| 394  | AAC AAG CCT GGC CCT ACT GTC GAG CAG ATC GAG AAT GCC TTC CAA GAG ACC CTC TGC     | 2503 | GAG ATG CTC ATA ACT GGT GGC AGA CAT CCC TTC CTC AAA TAC AAG GTG GGC TTC             |
| 151  | Arg Cys Thr Gly Tyr Arg Pro Ile Leu Gln Gly Phe Arg Thr Phe Ala Lys Asp Gly     | 854  | Met Lys Thr Gly Thr Ile Val Ala Leu Glu Val Ala His Phe Ser Asn Gly Gly Asn         |
| 453  | GCC TGT ACA GGC TAT AGA CCC ATC CTC GCG GGA TTC CCG ACC TTT CCG AAG GAT GGT     | 2582 | ATG AAG ACT GGC ACT ATA GTG GCA CTG GAG GTG GCA TTC ACC AAT GGC GGG AAC             |
| 170  | Gly Cys Ser Gly Gly Ser Glu Asn Asn Pro Asn Cys Cys Met Ser Gln Thr Lys Asp     | 873  | Ser Glu Asp Leu Ser Arg Ser Ile Met Glu Arg Gln Ala Val Phe His Met Asp Asn Ala     |
| 510  | GGG TGC TGT GGA GGA AGT GGA AAC AAC CCA AAC TGC TGC ATG AGC CAA ACA AAG GAC     | 2619 | ACT GAG GAT CTC TCT CCG AGT ATR ATG GAA AGA GCT GTA TTC CAG His Met Asp Asn Ala     |
| 189  | Gln Thr Ile Ala Pro Ser Ser Ser Leu Phe Asn Pro Glu Asp Phe Lys Pro Leu Asp     | 892  | Tyr Lys Ile Pro Asn Ile Arg Gly Thr Gly Arg Ile Cys Lys Thr Asn Leu Pro Ser         |
| 567  | CAG AAG ATT GCT CCC TCA TCT TTA TCC AAC CCG GAG GAT TTC AAA OCT TTA GAT         | 2676 | TAT AAG ATC CCC AAC ATT GCG GGC ACC GGG AGG ATT TGT AAG ACT AAT CTG CCC TCT         |
| 208  | Pro Thr Gln Glu Pro Ile Phe Pro Pro Glu Leu Leu Arg Leu Lys Asp Thr Pro Arg     | 911  | Asn Thr Ala Phe Arg Gly Phe Gly Gly Pro Gln Gly Met Leu Ile Ala Gly Tyr Trp         |
| 624  | CCC ACC CAA GAG CCC ATC TTT GGC CCA GAG TTG CTG AGG CTG AAA GAC ACT CCC GGG     | 2733 | AAC ACA GGC TTC AAG GGC TTT GGC GGT CCT CAG GGG ATG CTA ATC GCA GAA TAC TGG         |
| 227  | Lys Thr Leu Arg Phe Gly Arg Val Thr Ile Gln Thr Trp Ile Gln Ile Ser Thr Met Glu | 930  | Met Ser Glu Val Ala Val Thr Cys Gly Leu Pro Ala Glu Glu Val Arg Arg Lys Asn         |
| 681  | AAG ACG TTG COT TTT GAA GGG GAA COT GTG AOC TGC ATG ACT TTA ACC ATG GAG         | 2790 | ATG AOT GAG GTC GCT GTC ACC TGT GGC CTG OCT GCA GAG GTA ATG GGG AAG AAA AAC         |
| 246  | Glu Leu Leu Asp Leu Lys Ala Gln His Pro Asp Ala Lys Val Val Val Gly Asn Thr     | 949  | Met Tyr Lys Glu Gly Asp Leu Thr His Phe Asn Gln Lys Leu Glu Gly Phe Thr Leu         |
| 738  | GAA CTG CTT GAC CTC AAA GCG CAG CAC OCT GAT GCC AAG CTG GTG GGA AAC ACA         | 2847 | ATG TAC AAA GAA GGG GNC CTG ACT CAC TTC AAC CAG AAG CTG GAG GGG TTC ACC TTG         |
| 265  | Glu Ile Gly Ile Glu Met Lys Phe Lys Asn Met Leu Phe Pro Leu Ile Ile Cys Pro     | 968  | Pro Arg Cys Trp Asp Glu Cys Ile Ala Ser Ser Gln Tyr Gln Ala Arg Lys Met Glu         |
| 795  | GAG ATA GGC AAT GAA ATG AAA TTT AAA AAT ATG CTA TTT OCT CTG ATC ATC TGC CCA     | 2904 | CCC ACG TGC TCG GAT GAG TGC ATA GCG AGC TCC CAG TAT CAG GCT CCG AAG ATG GAA         |
| 284  | Ala Trp Ile Leu Glu Leu Thr Ser Val Ala His Gly Pro Glu Gly Ile Ser Phe Gly     | 987  | Val Glu Lys Phe Asn Arg Glu Asn Cys Trp Lys Lys Arg Gly Cys Lys Ile Ile Pro         |
| 852  | GCC TGG ATC CTT GAA CTG ACC TCA CTC GCA CAT GGG OCT GAG GCA ATC TCC TTT GGA     | 2961 | GTG GAG AAA TCT AAC AAG GAG AAC TGT TGC AAA AAG AGA GGG CTG TGT ATA CTC CCG         |
| 503  | Ala Ala Cys Pro Leu Ser Leu Val Glu Ser Val Leu Ala Asp Ala Ile Ala Thr Leu     | 1006 | Thr Lys Phe Gly Ile Ser Phe Thr Leu Ser Phe Leu Asn Gln Gly Gly Ala Leu Val         |
| 909  | GCC GCT TCC CCC CTT ACC TTG GTG GAA AGT GTC CTG CCG GAG CCG ATT GCT ACA CTT     | 3018 | ACT AAG TTT GGA ATA ACC TTC ACA CTT TCT TTT CTG AAC CAG GGG GGT GCT TTG GTC         |
| 322  | Pro Glu Gln Arg Thr Glu Val Phe Arg Gly Val Met Glu Gln Leu Arg Trp Phe Ala     | 1025 | His Val Tyr Thr Asp Gly Ser Val Leu Leu Thr His His Gly Gly Thr Glu Met Gly Gln     |
| 966  | CCA GAG CAG AGG ACA GAG GTC TTC AGA GGC GTG ATG GAG CAG CTG CCG TGG TTT GCT     | 3075 | CAC GTG TAC ACG GAT GGG TCG GTG CTG CTG ACA CAT GGA GGT ACT GAG ATG GGT CAA         |
| 341  | Gly Lys Gln Val Lys Ser Val Ala Ser Ile Gly Gly Asn Ile Ile Thr Ala Ser Pro     | 1044 | Gly Leu His Thr Lys Met Val Gln Val Ala Ser Arg Arg Ala Leu Lys Ile Pro Thr Ser     |
| 1023 | GGC AAG CAG CTG ACC TCC GCG GGC TCC ATT GGC GGG AAC ATC ATC ACC GGG AGC CCC     | 3132 | GCC CTF CCA ACC AAC ATG GTF GAG GTG GCG ACC AGC AGA GCT CTC AAA ATC CCC ACT TCC     |
| 360  | Ile Ser Asp Leu Asn Pro Val Leu Met Ala Ser Arg Ala Lys Leu Thr Leu Ala Ser     | 1063 | Lys Ile His Ile Thr Glu Thr Ser Thr Asn Thr Val Pro Asn Thr Ser Pro Thr Ala         |
| 1080 | ATC TCT GAC CTC AAG CCT GTG CTC ATG GCG AGT CGA GGC AAG CTG ACC CTC GCA TCT     | 3189 | AAG ATC CAG ATA ACG GAG ACA AGC ACT AAC ACT CTT CCA ACC CTT CCC ACG GCT             |
| 479  | Arg Gly Thr Lys Arg Thr Val Trp Met Asp His Thr Phe Phe Pro Gly Tyr Arg Arg     | 1082 | Ala Ser Ser Ala Asp Leu Asn Gly Gln Ala Ile Tyr Glu Ala Cys Gln Thr Ile             |
| 1137 | AGA GGT ACC AAG AGA ACG GTC TGG ATG GAC CAT ACC TTC TTC OCT GGC TAT AGA AAG     | 3246 | GCC TCT GCC ACG GCT GAC CTC AAT GGC CAG GCC ATT TAT GAA GCC TGT CAG ACC ATA         |
| 398  | Thr Leu Leu Ser Pro Glu Glu Ile Leu Ser Ile Val Ile Pro Tyr Ser Arg Lys         | 1101 | Leu Arg Tyr Ser Ala Glu Pro Phe Lys Lys Asn Pro Ser Gly Ser Trp Glu Ser Trp         |
| 1194 | ACT CTG CTC AOT CCA GAG CAG ATA TTG TGG TCC ATT GTG ATC CCC TAC ACG AAG AAG     | 3303 | CTG AAA AGA CTG GAG CCC TTC AAG AAA AAG AAT CCC TCA GCG TCC TGG GAG ACC TGG         |
| 417  | Gly Glu Phe Phe Ser Ala Phe Lys Gln Ala Ser Arg Arg Glu Asp Asp Ile Ala Lys     | 1120 | Val Met Asp Ala Tyr Thr Ser Ala Val Ser Leu Ser Ala Thr Gly Phe Tyr Lys Thr         |
| 1251 | GGT GAG TTT TTC TCA GCC TTC AAG CAG GCC TCC AGG AGA GAA GAT GAC ATT GCC AAG     | 3360 | GTG ATG GAT GCC TAC ACT AAT GCA GTG ACC TTG TCT GCT ACT GGA TTT TAT AAG ACA         |
| 436  | Val Thr Ser Gly Met Arg Val Leu Phe Lys Pro Gly Thr Thr Glu Val Gln Glu Leu     | 1139 | Pro Asn Leu Gly Tyr Ser Phe Glu Thr Asn Ser Gln Asn Pro His Thr Tyr Phe Ser         |
| 1309 | GTG ACC AOT GGC ATG AGA GTC CTC TTC AAG CCA GGC ACT GAA GTG GAG GAA CTG         | 3417 | CCC AAC CTT GGT TAC ACC TTT GAG ACA AAC TCT GGG AAC CCC TTC CAC TAC TTC AGT         |
| 455  | Ser Leu Cys Phe Gly Gly Met Ala Asp Arg Thr Val Ser Ala Leu Lys Thr Pro         | 1158 | Tyr Gly Val Ala Cys Ser Glu Val Glu Ile Asp Cys Leu Thr Gly Asp His Lys Asn         |
| 1365 | TCC CTT TGC TTT GGA GGG ATG OCT GAC AGA ACT GTC TCA GCC CTC AAG ACC ACT CCG     | 3474 | TAT GGG GTG OCT TGC TCA GAA GTA GAA ATC GAG TGC TTA CCA GGG CAG CAT AAG AAT         |
| 474  | Lys Gln Leu Ser Lys Ser Trp Asn Glu Glu Leu Gln Asp Met Cys Ala Gly Leu         | 1177 | Leu Arg Thr Asp Ile Val Met Asp Val Gly Ser Ser Leu Asn Pro Ala Ile Asp Ile         |
| 1422 | AAG CAG CTG TCC AAG TCC TGC AAT GAG GAG TTG CTG CAG GAT GTG TGT GCT GGC TTC     | 3531 | CTG OCT CCA GAT ATC GTC ATG GAT GTT GCT OCT GAG CAA AAT CCT GCT ATT GAT ATC         |
| 493  | GCA Glu Gln Glu His Leu Ala Pro Asp Ala Pro Gly Gly Met Val Glu Phe Arg Arg     | 1196 | Gly Gln Val Glu Gly Ala Phe Val Gln Gly Leu Gly Leu Thr Met Glu Glu Leu Cys         |
| 1479 | AGA GAG GAG CTG CAC CTC GCG CCC GGC OCT GGT GGC ATG GTG GAA TFC CCG GCG         | 3588 | GSA CAG GTA GSA GGG GCA TTC GTC CAG GGT CTT GGT CTT TCC ACC ATG GAG GAG CTG         |
| 512  | Thr Leu Thr Leu Ser Phe Phe Phe Lys Phe Tyr Leu Thr Val Leu Gln Lys Leu Gly     | 1215 | His Tyr Ser Pro Glu Gly Ser Leu His Thr Ser Arg Gly Pro Ser Thr Tyr Lys Ile Pro     |
| 1536 | ACC CTC ACC CTC ACC TTC TTC TAC TAC CTC ACA GTG CTT CAG AAG CTG GGC             | 3645 | CAC TAC TCT CCC GAG GGC ACC CTC CAC ACT CBT GCG CCC AGT ACC TAC AAG CTC CTT         |
| 531  | Arg Ala Asp Leu Glu Gly Met Cys Gly Lys Leu Asp Pro Thr Phe Ala Ser Ala Thr     | 1234 | Ala Phe Gly Ser Ile Pro Ile Glu Phe Arg Val Ser Leu Val Arg Asp Cys Pro Asn         |
| 1593 | AGA GGC GAC CTT GAG GGT ATG TGT GGT AAA CTG GAC CCC ACC TTT GGC AGC GCC ACC     | 3702 | GCA TTT GGC AGC ATC CCC ATT GAG TTC AGA GTA TCC CTC GTC CCG GAG TGC CCC AAC         |
| 580  | Leu Leu Phe Gln Lys Asp Pro CCA Gln Asn Val Gln Leu Phe Gln Glu Val Pro Lys     | 1253 | Arg Arg Ala Ile Tyr Ala Ser Lys Ala Val Gly Ala Val Gly Gln CCA Pro Thr Leu Ala Ser |
| 1650 | CTG CTC TTT GAG AAG GAT CCT CCA GTC CAG CTT TTC CAA GAG GTG CCC AAG             | 3759 | AAG ACG GCG ATC TAT GCA TCC AAG GCT GTC GGC GAG CUA CCT CTT TTC CTG GCT TCT         |
| 549  | Gly Gln Ser Glu Glu Asp Met Val Gly Arg Pro Met Pro His Leu Ala Ala Asp Met     | 1272 | Ser Ile Phe Phe Ala Ile Lys Asp Ala Ile Arg Ala Ala Arg Ala Gln His Gly Asp         |
| 1717 | GGT GAG TCT GAG GAG CAG ATG GTC AGG CCC ATG CCT CAC CTG GCA GCA GAG ATG         | 3816 | TGG ATC TTT GCT TTT GCT ATC AAA GAT GCT ATC CCG GCA GCT CGA GCT CAG GGA GAG         |
| 588  | Gln Ala Ser Gly Glu Ala Val Tyr Cys Asp Asp Ile Pro Arg Tyr Glu Asn Glu Leu     | 1291 | Ser Asn Ala Lys Gln Leu Phe Gln Leu Asp Ser Pro Ala Thr Pro Glu Lys Ile Arg         |
| 1764 | CAG GCA TCC GGG GAG GCT CTG TAC TGT GAT GAC ATT CCG CCG TAT GAG AAT GAG CTG     | 3873 | AOT AAC GGC AAA CAG CTC TTC CAG CTA GAC AGC CCC GGC ACT CCG GAG AAG ATC CCA         |
| 607  | Ser Leu Arg Leu Val Thr Ser Thr Arg Ala His Ala Lys Ile Met Ser Ile Asp Thr     | 1310 | Asn Ala Cys Val Asp Gln Phe Thr Thr Leu Cys Ala Thr Gly Thr Pro Glu Asn Cys         |
| 1827 | TCC CTC AAG CTG GTC ACC AGC ACG CGG GCC CAT GCT AAA ATC ATG TCC ATC GAC ACT     | 3930 | AAC GCT TGT GTC GAT CAG TTC ACC ACC CTG TGT GCT ACC TTA CCA CCA GAA AAC TGT         |
| 626  | Ser Glu Ala Lys Lys Val Pro Gly Phe Val Cys Phe Leu Thr Ser Glu Asp Val Pro     | 1329 | Lys Ser Trp Ser Val Arg Ile Ter   |
| 1878 | TCA GAA GGC AAG AAG CTG CCA GGG TTT GFT TGT TTC CTC ACC TCA GAG GAT GTC CCT     | 3987 | AAA TCC TGC TCC GTG AGC ATC TGAAGAGGAGCCCTCCAGTATGGTTTTACTACAGCCCGGATTCCTC          |
| 645  | Gly Ser Asn Ile Thr Gly Ile Phe Asn Asp Thr Val Phe Ala Lys Asp Glu Val         | 4057 | GGAGATGCAAGTCATCCGATATCCAGATTTCCACACATCCCATGGGGACTCAGCAGGATGGCAATTTTTCAGAA          |
| 1935 | GGT AGT AAC ATA ACT GGC ATT TTC AAT GAT GAA ACT GTC TTT GCG AAG GAT GAG GTT     | 4134 | AATGGCCATTTGATCCAAATTCATGATTTCCACACACAAACCAATAGCAAAAGGGGACTCTGCGCCAGTCGAG           |
| 648  | Thr Cys Val Gly Phe Ile Ile Gly Ala Val Ile Asp Thr Val Glu His Ala His         | 4211 | TTTGTAGTAATTCGGGTAACTCTGATCCATTTTGTACACATATTTGAAATGAGGTTAGCAGGGTTTCGGTTAT           |
| 1942 | ACT TGT CTT GGG TAC ATC ATT GGT OCT GTC GCT GAC ACC CCA GAA CAT GCA CAC         | 4288 | GTCCCTACTCCACATGCGGCTGTTACTAGATTCCTCCACACCTCTCCACCCGTAACCAACCGTTCCTAGAT             |
| 689  | Arg Ala Ala Arg Gly Val Lys Ile Thr Tyr Glu Asp Leu Pro Ala Ile Ile Thr Ile     | 4365 | CTCCATGCTTCTTCAGAGCTTATCTGCCCAGCCCAACAGGGCCCGTGTGTAAACGCGACTGCACACTATAG             |
| 2049 | AGA GGC GCT ACG GCG CTG AAA ATC ACC TAT GAA GAC CTT CCA CCC ATT ATC ACA ATC     | 4412 | TCTCAAGTAAATGTTGTGTGTATCTGTGTGGGGGGGGGGGATAAAACCTTCCACATCCCTTATCATATAGAA            |
|      |   | 4429 | GGATGAAACCTGATATGAAAAAATAAAAAAAAAAAAAA  |

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<sup>+</sup>-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<sup>+</sup> 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.

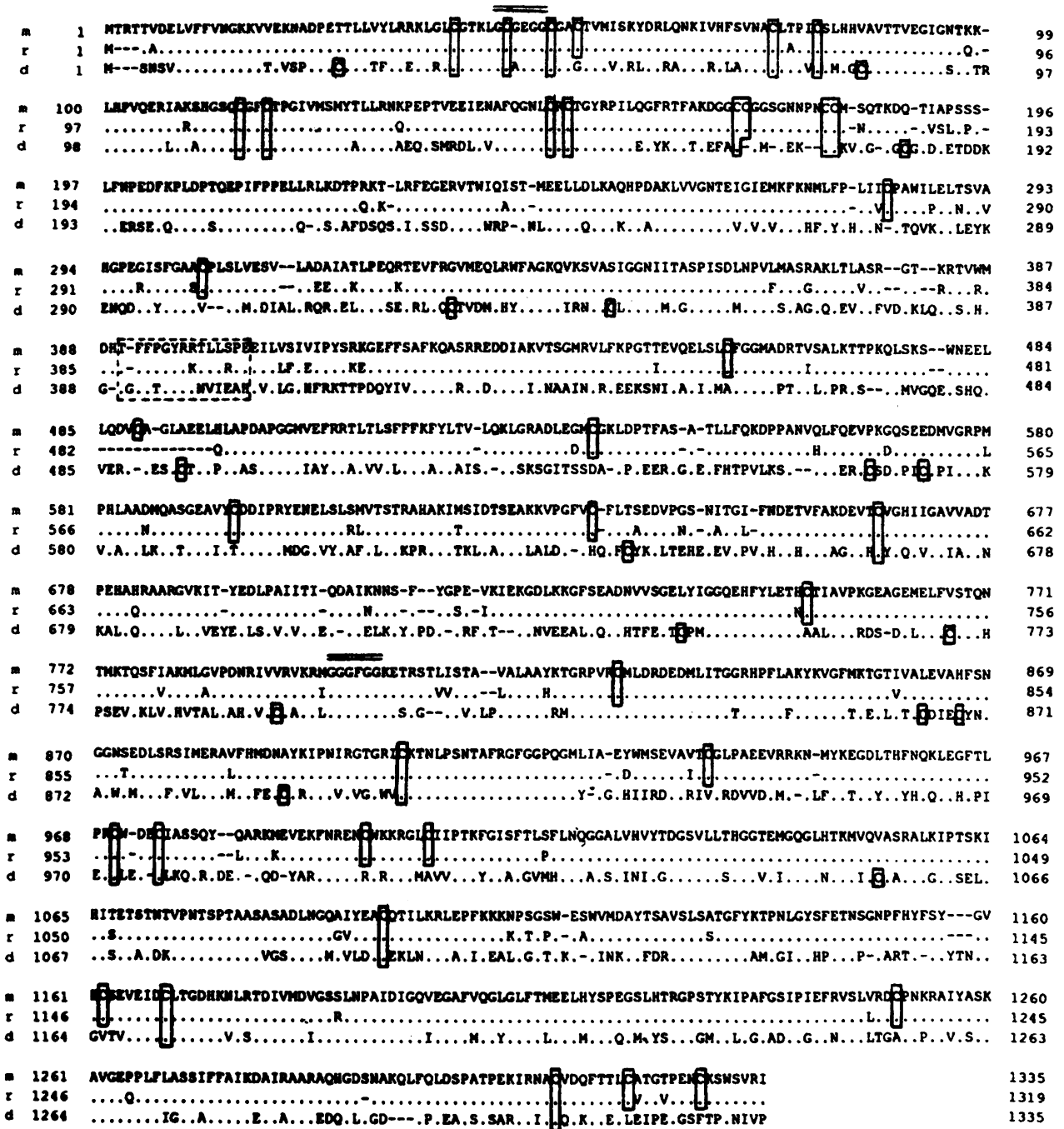


Fig. 3. Amino acid sequence comparison with rat and *Drosophila melanogaster* XD

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<sup>+</sup> or FAD, are doubly underlined. The region corresponding to the binding site of NAD<sup>+</sup>, as determined by comparison of the 5'-*p*-fluorosulphonylbenzoyladenine-labelled peptide sequence of chicken XD (Nishino & Nishino, 1989), is boxed by a dotted line.

difference in the environment at the NAD<sup>+</sup>-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<sup>+</sup> 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

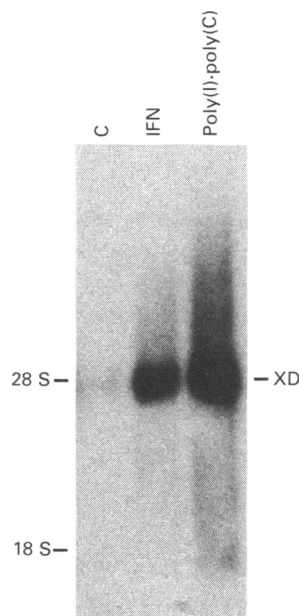


Fig. 4. Induction of mouse liver XD mRNA by IFN- $\alpha$  and poly(I)·poly(C)

Polyadenylated RNA (5  $\mu$ g/each lane) was extracted from the liver of two mice treated with saline (C),  $1.5 \times 10^5$  units of IFN- $\alpha$  (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.

was extracted from mouse liver before and after treatment with IFN- $\alpha$  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- $\alpha$  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. 5a). At 24 h, the level of XD mRNA decreases towards the basal level. To study whether XD mRNA correlates with its translation into catalytically 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. 5b). 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  $\pm$  s.e.m. is  $1.2 \pm 0.1$  in control conditions compared with  $1.1 \pm 0.2$  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- $\alpha$  are

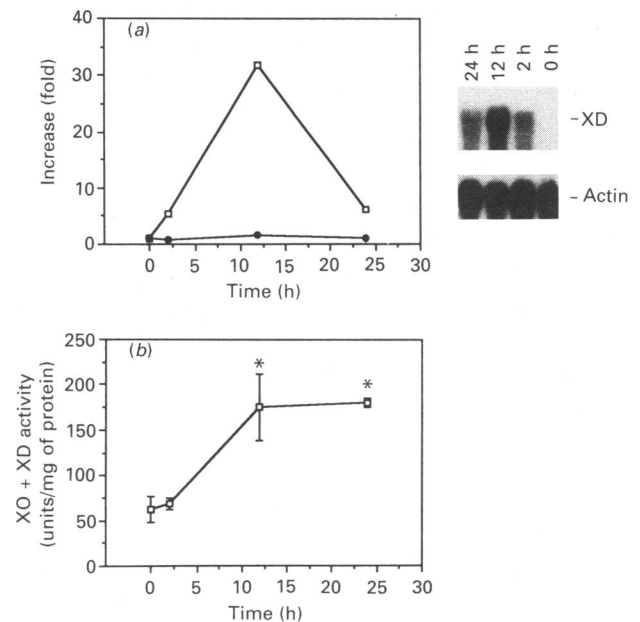


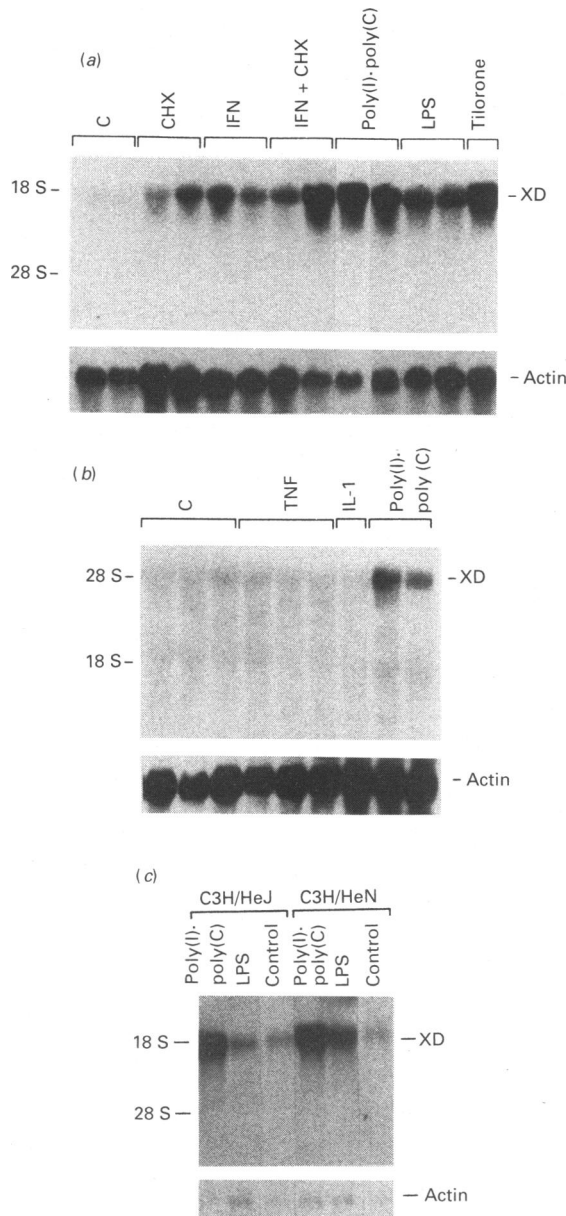
Fig. 5. Time course of the induction of XD mRNA and XO+XD activity in the liver of mice treated with poly(I)·poly(C)

(a) Total RNA (20  $\mu$ g 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.  $\square$ , XD mRNA;  $\bullet$ , 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  $\pm$  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).

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- $\alpha$  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- $\alpha$ , demonstrating that protein synthesis *de novo* is not required for this induction.

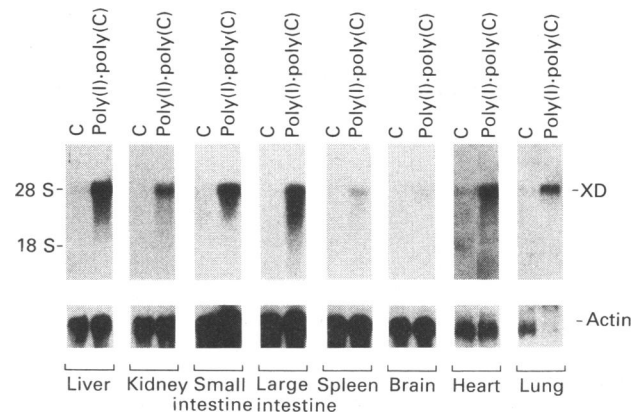
Since LPS is known to stimulate many other cytokines besides IFN- $\alpha$  *in vivo*, the specificity of the elevation of XD message by IFN- $\alpha$  was examined. Animals were treated with two cytokines known to be induced by LPS *in vivo*, i.e. tumour necrosis factor and interleukin-1. Fig. 6(b) 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. 6c).

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 X-D mRNA in the mouse liver

(a) The induction of X-D 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<sup>5</sup> 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 X-D mRNA induction was also tested. Each lane represents the result from a single animal. Filters were sequentially hybridized to X-D 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 X-D 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 X-D 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 X-D 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 X-D 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 X-D and actin cDNAs.



**Fig. 7.** Induction of X-D 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 X-D and actin cDNAs.

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

This specific induction places X-D 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 X-D 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 I recombinant IFN-α, but induction of X-D 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 X-D mRNA by the two types of IFNs requires the same or different intracellular signals.

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

As to the role of the X-D/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<sub>2</sub><sup>-</sup> anions derived from its activity as the mediators of this toxic effect. A key question is represented by the role of the X-D/XO system in other biological effects produced by IFNs. It is possible that the increased concentration of X-D 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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## REFERENCES

- Amaya, Y., Yamazaki, K., Sato, M., Noda, K., Nishino, T. & Nishino, T. (1990) *J. Biol. Chem.* **265**, 14170–14175
- Bender, W., Spierer, P. & Hogness, D. S. (1983) *J. Mol. Biol.* **168**, 17–33
- Bindoli, A., Valente, M. & Cavallini, L. (1985) *Pharm. Res. Commun.* **17**, 831–839
- Bindoli, A., Cavallini, L., Rigobello, M. P., Coassim, M. & Lisa, F. D. (1988) *Free Radical Biol. Med.* **4**, 163–167
- Birnstiel, M. L., Busslinger, M. & Strub, K. (1985) *Cell* **41**, 349–359
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Brass, C. A., Narciso, J. & Gollan, J. L. (1991) *J. Clin. Invest.* **87**, 424–431
- Carpani, G., Racchi, M., Ghezzi, P., Terao, M. & Garattini, E. (1990) *Arch. Biochem. Biophys.* **279**, 237–241
- de Groot, H. & Littauer, A. (1988) *Biochem. Biophys. Res. Commun.* **155**, 278–282
- Della Corte, E. & Stirpe, F. (1968) *Biochem. J.* **108**, 349–351
- Della Corte, E. & Stirpe, F. (1972) *Biochem. J.* **126**, 739–745
- Deloria, L., Abbott, V., Gooderham, N. & Mannering, G. J. (1985) *Biochem. Biophys. Res. Commun.* **131**, 109–114
- Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., pp. 203–204. Academic Press, New York
- Elsayed, N. M. & Tierney, D. F. (1989) *Arch. Biochem. Biophys.* **273**, 281–286
- Engerson, T. D., McKelvey, T. G., Rhyne, D. B., Boggio, E. B., Snyder, S. J. & Jones, H. P. (1987) *J. Clin. Invest.* **79**, 1564–1570
- Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13
- Fridovich, I. (1970) *J. Biol. Chem.* **245**, 4053–4057
- Ghezzi, P., Bianchi, M., Mantovani, A., Spreafico, F. & Salmona, M. (1984) *Biochem. Biophys. Res. Commun.* **119**, 144–149
- Ghezzi, P., Bianchi, M., Gianera, L., Landolfo, S. & Salmona, M. (1985) *Cancer Res.* **45**, 3444–3447
- Gilbert, D. A. & Bergel, F. (1964) *Biochem. J.* **90**, 350–353
- Granger, D. N., Rutili, G. & McCord, J. M. (1981) *Gastroenterology* **81**, 22–29
- Guest, J. R. & Rice, D. W. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C. & Mayhew, S. G., eds.), pp. 111–124. Walter de Gruyter, Berlin
- Huynh, T. V., Young, R. A. & Davis, R. W. (1985) in *DNA Cloning: A Practical Approach* (Glover, D. M., ed.), vol. 1, pp. 49–78. IRL Press, Oxford
- Jung, V., Jones, C., Rashidbaigi, A., Geyer, D. D., Morse, H. G., Wright, R. B. & Pestka, S. (1988) *Somat. Cell. Mol. Genet.* **14**, 583–592
- Jung, V., Jones, C., Kumar, C. S., Stefanos, S., O'Connell, S. & Pestka, S. (1990) *J. Biol. Chem.* **265**, 1827–1830
- Keith, T. P., Riley, M. A., Kreitman, M., Lewontin, R. C., Curtis, D. & Chovnick, A. (1987) *Genetics* **116**, 67–73
- Krenitsky, T. A., Spector, T. & Hall, W. W. (1986) *Arch. Biochem. Biophys.* **247**, 108–119
- Lee, C. S., Curtis, D., McCarron, M., Love, C., Gray, M., Bender, W. & Chambers, G. (1987) *Genetics* **116**, 55–66
- Levy, D. E., Kessler, D. S., Pine, R., Reich, N. & Darnell, J. E., Jr. (1988) *Genes Dev.* **2**, 383–393
- Mariatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Mannering, G. J., Deloria, L. B. & Abbott, V. (1988) *Cancer Res.* **48**, 2107–2112
- McCord, J. M. (1985) *N. Engl. J. Med.* **312**, 159–163
- 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
- Nishino, T. & Nishino, T. (1989) *J. Biol. Chem.* **264**, 5468–5473
- O'Brien, A. D., Metcalf, E. S. & Rosenstreich, D. L. (1982) *Cell Immunol.* **67**, 325–333
- Oda, T., Hamamoto, T., Suzuki, F., Hirano, T. & Maeda, H. (1989) *Science* **244**, 974–976
- Parks, D. A. & Granger, D. N. (1983) *Am. J. Physiol.* **245**, G285–G289
- Rehberg, E., Kelder, B., Hoal, E. G. & Pestka, S. (1982) *J. Biol. Chem.* **257**, 11497–11502
- Reiners, J. J., Pence, B. C., Barcus, M. C. S. & Cantu, A. R. (1987) *Cancer Res.* **47**, 1775–1779
- Renton, K. W., Singh, G. & Stebbing, N. (1984) *Biochem. Pharmacol.* **33**, 3899–3902
- Roy, R. S. & McCord, J. M. (1982) *Can. J. Physiol. Pharmacol.* **60**, 1346–1352
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Staheli, P. (1990) *Adv. Virus Res.* **38**, 146–200
- Stirpe, F. & Della Corte, E. (1969) *J. Biol. Chem.* **244**, 3855–3863
- Suleiman, S. A. & Stevens, J. B. (1985) *Arch. Biochem. Biophys.* **258**, 219–225
- Taylor, M. D., Mellert, T. K., Parmentier, J. L. & Eddy, L. J. (1980) *Brain Res.* **347**, 268–273
- Terada, L. S., Rubinstein, J. D., Lesnefsky, E. J., Horwitz, L. D., Leff, J. A. & Repine, J. E. (1991) *Am. J. Physiol.* **260**, H805–H810
- Wickens, M. & Stephenson, P. (1984) *Science* **226**, 1045–1051
- Wierenga, R. K., De Maeyer, M. C. H. & Hol, W. G. J. (1985) *Biochemistry* **24**, 1346–1357
- Williams, C. H., Jr., Arscott, L. D. & Swenson, R. P. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C. & Mayhew, S. G., eds.), pp. 95–109. Walter de Gruyter, Berlin
- Wood, W. I., Critschier, U., Lasky, L. A. & Lawn, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1585–1588

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