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Isolation and characterization of the rat tryptophan oxygenase gene

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Tryptophan oxygenase (TO, EC 1.13.1.12) from rat liver is subject to glucocorticoid and developmental control. To study the mechanism of regulation, TO mRNA sequences and the chromosomal TO gene were cloned. From a cDNA library prepared from rat liver poly(A)$^+$ RNA enriched for TO mRNA, a recombinant plasmid containing TO cDNA sequences was identified by translation of hybrid-selected RNA and immunoprecipitation with antibodies directed against TO. This cDNA clone hybridizes to a mRNA 2000 bases long that is inducible by dexamethasone. With this clone as probe we isolated from a bacteriophage λ rat DNA library genomic clones which together span a region of 32 kilobase pairs (kb). Heteroduplex analysis revealed that the gene extends over 19 kb and is interrupted by at least 11 introns. To characterize the presumptive control region the DNA sequence around the 5′ end of the TO gene was determined. S1 nuclease protection experiments revealed two separate start sites for TO mRNA transcription within this region.

Key words: exon-intron structure/glucocorticoid control/multiple mRNA start sites/regulatory sequences

Introduction

Steroid hormones affect various physiological functions in their target tissues by induction of specific proteins. Classical examples are the increase in enzymatic activity of tyrosine aminotransferase (TAT) and tryptophan oxygenase (TO) in rat liver following glucocorticoid administration (Knox and Mehler, 1951; Lin and Knox, 1958). The increase in activity of these two gluconeogenic enzymes is the result of an increased rate of synthesis of the enzyme proteins (Feigelson and Greengard, 1962; Schimke *et al.*, 1965; Kenney, 1962). The glucocorticoid induction of TO and TAT in rat liver and hepatoma cells has therefore become a suitable model system for effector-mediated control of gene expression in mammalian cells.

Quantitation of the mRNA levels for these two enzymes by *in vitro* translation in cell-free protein synthesizing systems revealed that the rise in enzyme synthesis results from an increase in the amount of translatable TO and TAT mRNA produced after hormone administration (Schütz *et al.*, 1973, 1975; Röwekamp *et al.*, 1976). The observed increase in the amounts of functional TO and TAT mRNA following induction by steroids indicate that control of enzyme induction in these two cases is pre-translational and most probably results from a hormonal enhancement of transcription. That regulation in fact occurs at this level has now been shown by us for TO by measuring nuclear transcription rates after hormone induction (Danesch *et al.*, in preparation).

In addition to hormonal modulation, both TO and TAT activity are subject to developmental control. TAT enzyme activity first appears around birth (Greengard, 1970), whereas TO enzyme activity (Nemeth, 1959; Franz and Knox, 1967) as well as translatable TO mRNA are first detected around the fifteenth post-natal day, increasing to the adult level within a week (Killewich and Feigelson, 1977).

The mechanisms by which steroids regulate TO and TAT mRNA synthesis are unknown, as are the control mechanisms operating during development. We expect that a comparative analysis of the structure and function of these two genes will provide some insight into these control processes. Because an understanding of gene regulation at the molecular level requires specific DNA probes, we have isolated the TO and TAT genes. Here we describe the construction of a TO cDNA clone and its use in isolating genomic clones containing the TO structural gene. The recently isolated TAT cDNA clones will be described elsewhere.

Results

Isolation of a rat TO cDNA clone

Rats were injected with the glucocorticoid dexamethasone

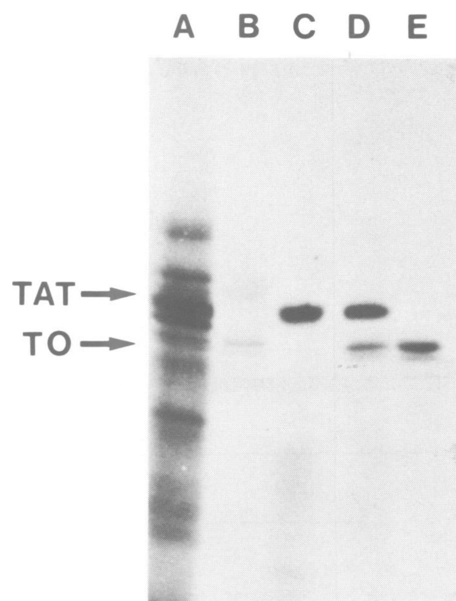

Fig. 1. Identification of a TO cDNA clone by immunoprecipitation of translation products of hybrid-selected mRNA. **Lane A**: *In vitro* translation products from poly(A)$^+$ RNA isolated from livers of dexamethasone-induced rats; **lane B**: same as **lane A** after immunoprecipitation with TAT- and TO-antibodies; **lane C**: products endogenous to the reticulocyte lysate; **lane D**: products from mRNA hybridized to pcTO-1; **lane E**: same as **lane D** after immunoprecipitation with TO-antibodies. Approximately five times the amount of the total translation products shown in **lanes A** and **D** were used for the immunoprecipitations shown in **lanes B** and **E**, respectively.

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and 4 h later, when the level of TO mRNA is at its maximum (Röwekamp *et al.*, 1976), polysomal RNA was prepared as starting material for TO mRNA isolation. Since maximally induced TO mRNA levels are estimated to represent only ~0.1% of total hepatic poly(A)$^+$ RNA (Killewich and Feigelson, 1977), we enriched ~10-fold for TO mRNA by two rounds of column chromatography (see Materials and methods). TO mRNA activity was assayed by *in vitro* translation and immunoprecipitation with TO-specific antiserum. The enriched TO mRNA was used as a template for the synthesis of double-stranded cDNA, which was inserted into the *Pst*I site of pBR322, using the polydG.polydC tailing technique. 750 tetracycline-resistant, ampicillin-sensitive clones were produced.

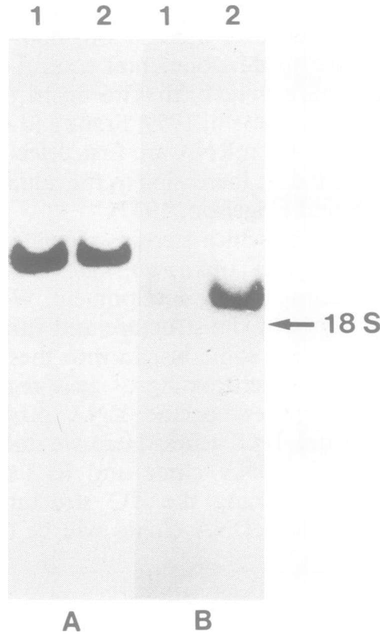


Fig. 2. Clone pcTO-1 hybridizes to a glucocorticoid-inducible mRNA. Poly(A)$^+$ RNA (1 μg) from livers of adrenalectomized **(1)** or dexamethasone-induced **(2)** rats was separated in duplicate on a 1% agarose formaldehyde gel, transferred to a nitrocellulose filter and hybridized with ^{32}P-labelled probes. **A**: Hybridization with an albumin cDNA clone. **B**: Hybridization with the pcTO-1 probe. The position of rat 18S rRNA (1940 bases; McMaster and Carmichael, 1977) is indicated by an arrow.

A plasmid carrying a TO cDNA insert was identified by translation of hybrid-selected RNA. Plasmid DNA from 250 clones was prepared, pooled in groups of 10, bound to nitrocellulose filters, and hybridized to poly(A)$^+$ RNA extracted from livers of dexamethasone-induced rats. Hybridized RNA was eluted and translated in a reticulocyte protein synthesizing system. From one positive pool we isolated the plasmid pcTO-1 which selected a mRNA that directed the synthesis of a protein of the size of TO (Figure 1, lane D). This protein was positively identified as TO by immunoprecipitation with antibodies against TO (Figure 1, lane E). The faint band visible in the immunoprecipitate lanes just below TO most likely results from premature termination of translation of TO mRNA. Since this band is found in immunoprecipitates of translation products from hybrid-selected RNA we can rule out an impurity of our antibodies.

Northern blot analysis showed that the plasmid pcTO-1 contains sequences complementary to a hormone-inducible mRNA species (Figure 2B). As a control, parallel lanes of the same blot were probed for albumin mRNA, which is not induced by glucocorticoids (Bélanger *et al.*, 1981) (Figure 2A). The estimated size of ~2000 bases for the TO mRNA, which runs between albumin mRNA (2150 bases; Sargent *et al.*, 1981) and 18S rRNA (1940 bases; McMaster and Carmichael, 1977) (Figure 2), is in good agreement with earlier estimates (Hofer and Sekeris, 1977).

Rescreening of the 750 recombinant plasmids by hybridization with either the ^{32}P-labelled 560-bp insert of pcTO-1 or with cloned genomic fragments (see below) resulted in identification of four additional TO clones. Thus, 5/750 or 0.7%

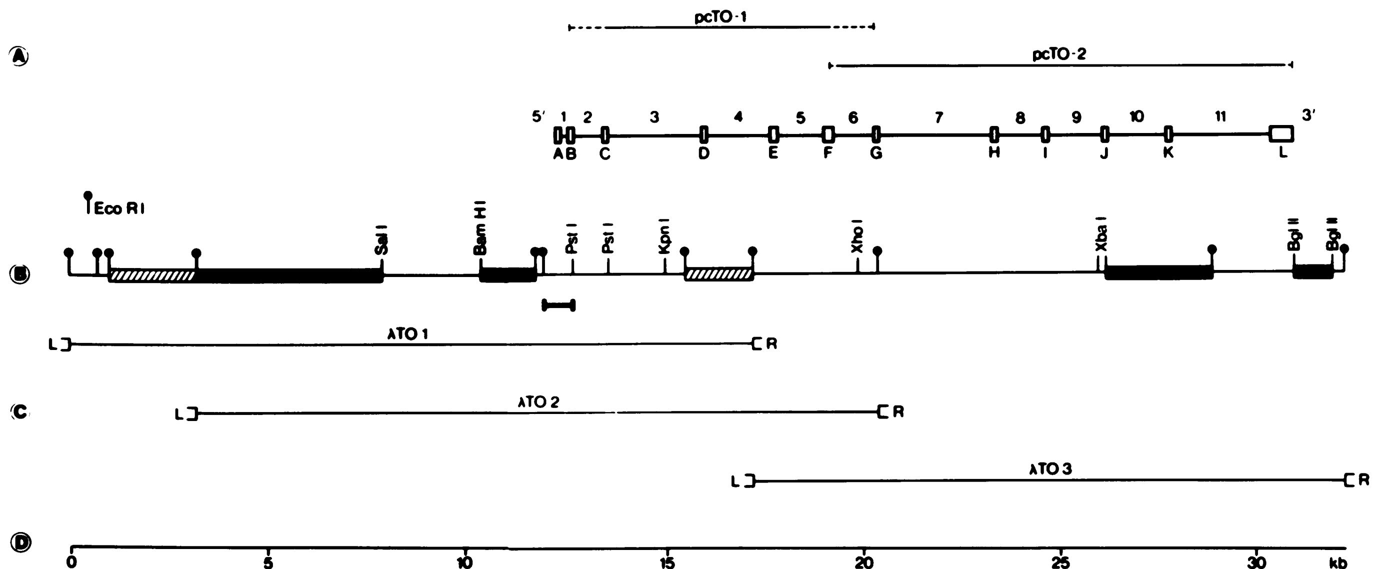
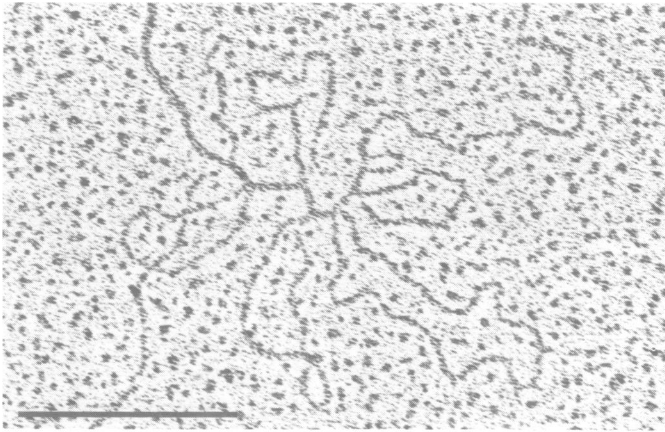
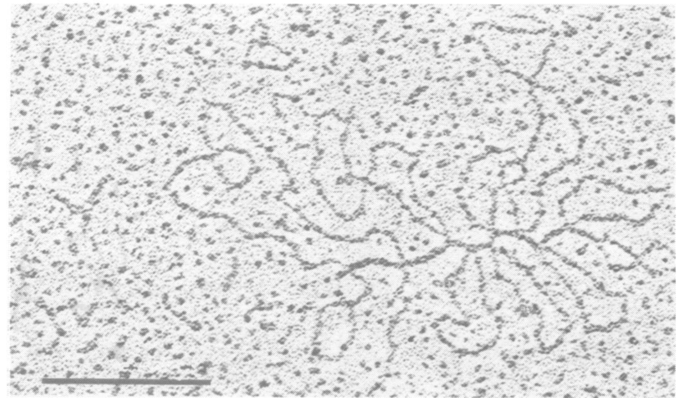
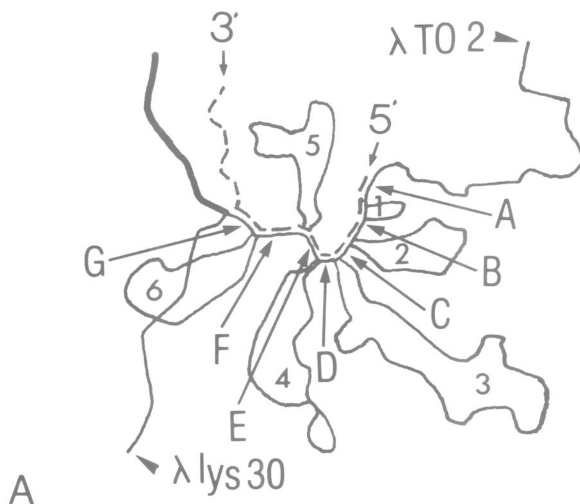


Fig. 3. Organization of the rat TO gene. **(A)** Location of the exons (A – L, open boxes) and introns (1 – 11) as determined by the heteroduplex experiments shown in Figure 4. The approximate alignment of the two cDNA clones pcTO-1 and pcTO-2 is indicated. **(B)** Map of restriction sites. The exact order of the three left-most *Eco*RI fragments is not known. For *Bam*HI and *Pst*I, only the sites around the 5′ end of the TO gene are indicated. |——|: region at the 5′ end of the gene whose sequence is presented in Figure 5. Restriction fragments containing repetitive sequences and hybridizing to labelled rat liver DNA (filled bars) and in addition to clone pRB1 containing a rat Alu sequence equivalent (hatched bars) are shown. **(C)** Rat chromosomal DNA sequences cloned in three overlapping λ phage recombinants. The left and right arms of the λ Charon 4A vector are indicated by L and R, respectively. **(D)** Scale in kb.







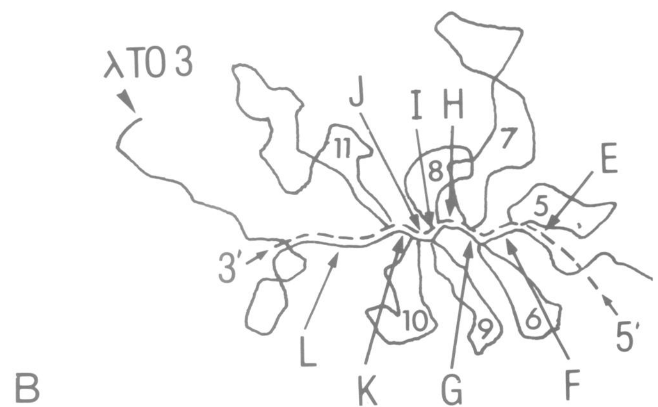


Fig. 4. Electron microscopy of RNA-DNA hybrids. Electron micrographs of hybrids formed between TO mRNA and the DNA of λ TO 2 and λ lys 30 (**A**) and λ TO 3 (**B**). In the interpretive drawings, RNA sequences are represented by a dashed line and DNA single- and double-stranded regions by thin and thick solid lines, respectively. The 5′ and 3′ termini of the TO mRNA are indicated. The lengths of exons A to L and intronic loops 1 – 11 are presented in Table I. The bar corresponds to 0.1 μm.

Table I. Length of intron/exon segments of the rat TO gene

	Exons	n		Introns	n
A	124 ± 38	7	1	223 ± 46	12
B	131 ± 35	11	2	872 ± 109	14
C	108 ± 22	15	3	2490 ± 250	15
D	102 ± 22	17	4	1628 ± 236	20
E	149 ± 25	20 + 6[a]	5	1253 ± 128	20 + 10[a]
F	209 ± 39	16 + 10[a]	6	1010 ± 113	16 + 14[a]
G	118 ± 24	10 + 14[a]	7	2924 ± 149	14
H	110 ± 20	12	8	1218 ± 128	13
I	90 ± 23	11	9	1407 ± 143	12
J	110 ± 23	8	10	1496 + 125	8
K	106 ± 26	8	11	2546 ± 216	5
L	518 ± 42	11			
Total	1875 ± 98			17 067 ± 532	

The exon/intron notation refers to Figures 3 and 4. The lengths in bases ± s.d. represent the averages of measurements of n heteroduplex molecules. The single- and double-stranded segments in hybrid molecules formed between the DNA of λ lys 30 and λ TO 2 or λ TO 3 (see Materials and methods) were used as internal standards. a: exons E to G and introns 5 and 6 were measured on both types of heteroduplex molecules (see Figure 4A and B).

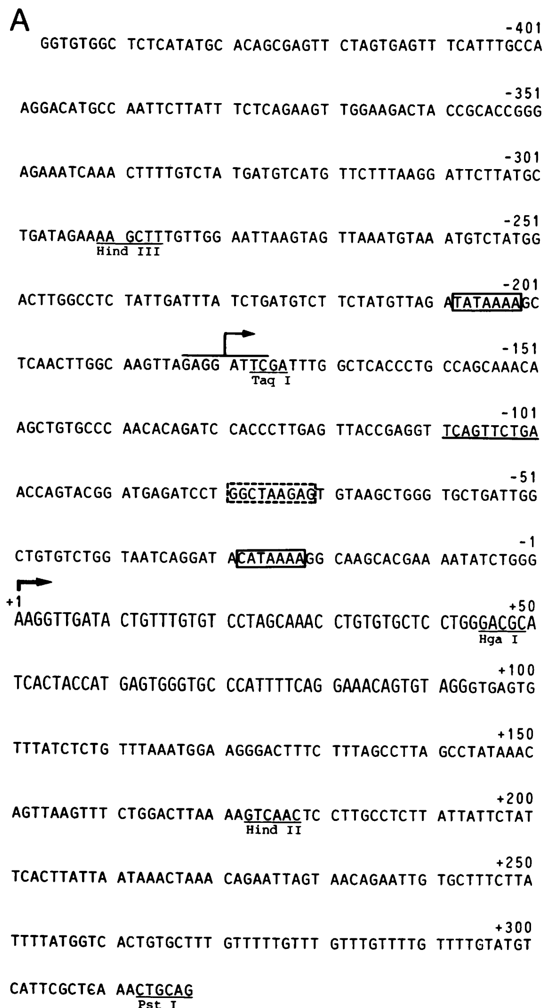
of our cDNA clones contained TO sequences, close to the 1% expected from an RNA fraction 10-fold enriched for TO mRNA.

Isolation and characterization of genomic TO clones

Radiolabelled pcTO-1 DNA was used to screen a phage library of rat liver DNA generated by partial digestion with *Eco*RI (Sargent *et al.*, 1979). From 450 000 recombinant phages, nine phages were independently isolated which represent five different but overlapping clones. The combined restriction map of three clones, which together cover a region of 32 kb and most likely contain the entire TO gene, is shown in Figure 3B and C.

The structure of the TO gene was determined by analysis of heteroduplexes formed between the DNA of genomic clones λTO 2 or λTO 3 and rat liver poly(A)$^+$ RNA enriched for TO mRNA. Representative electron micrographs with corresponding interpretations are presented in Figure 4.

The resulting structural map of the TO gene is shown in Figure 3A in relation to the restriction map of the gene. The direction of transcription was initially established by determining which strand of the cDNA insert in clone pcTO-1 is protected from S1 nuclease digestion by TO mRNA (not shown) and later confirmed by the S1 mapping experiments



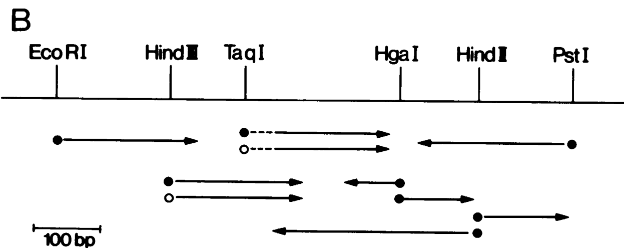


Fig. 5. Nucleotide sequence of the 5′ end region of the rat TO gene. **A**: DNA sequence of the *Eco*RI-*Pst*I fragment at the 5′ end of the TO gene (see Figure 3). The large arrow at position +1 indicates the major start site for TO gene transcription, while the minor start site around position −180 is indicated by the small arrow (see Figures 6 and 7). The first exon (+1 and +93) is given in large letters. The TATA sequences and the first ATG triplet in exon A are boxed. The sequence homology to the CAAT sequence is indicated by a dashed box. A possible regulatory site at position −101 to −110 as discussed in the text is underlined. Restriction sites used for sequencing are indicated. Only the non-coding strand is shown. **B**: Sequencing strategy used. DNA fragments were labelled either at their 5′ ends (●) or at their 3′ ends (○). The direction and extent of sequence determined are indicated by horizontal arrows. All sequence autoradiograms were analyzed independently by two investigators.

described below. The positions of the exons relative to the restriction enzyme sites have been confirmed by Southern blot analysis of the genomic clones probed with *in vitro* labelled rat liver poly(A)$^+$ RNA or with TO cDNA clones (not shown). As shown in Figure 3, the TO gene extends over 19 kb and contains at least 11 introns. The heteroduplex

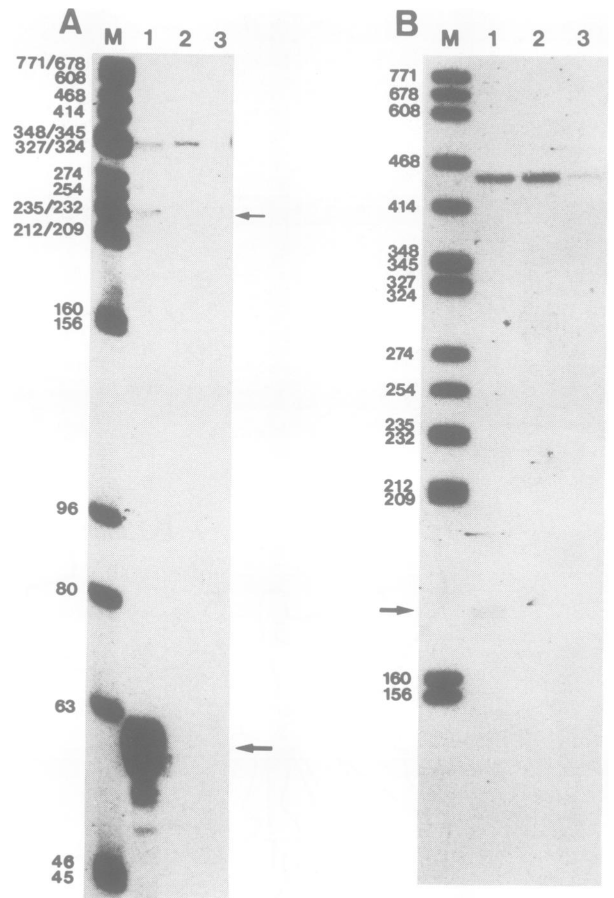


Fig. 6. S1 mapping of TO mRNA start sites with exon and intron probes. **A**: Exon probe. **Lane 1**: S1-resistant fragments obtained from hybrid between rat liver total poly(A)$^+$ and the 347-bp fragment *Hind*III-*Hga*I (recognition sites at −292 and +45, Figure 5A), 5′-labelled at the *Hga*I cleavage site at position +59. **Lane 2**: same as **lane 1**, except that yeast tRNA was incubated with the probe. **Lane 3**: same as **lane 1**, except that no RNA was added. M: radioactive pBR322 *Hinf*I marker fragments. The major and minor bands discussed in the text are indicated by a large and a small arrow. **B**: Intron probe. **Lane 1**: S1-resistant products obtained from hybrid between rat liver total poly(A)$^+$ RNA and the 465-bp *Hind*III-*Hind*II fragment (−292 to +173, Figure 5A), 5′-labelled at the *Hind*II site. **Lane 2, lane 3**, M: same as in **A**. Arrow points to S1-protected fragment.

measurements (Table I) indicate a total length for the 12 exons of 1875 ± 98 bp which agrees well with the above size estimate for the TO mRNA of ~2000 bases, allowing for a poly(A) tail of 100 bases.

Within the cloned 32 kb of rat DNA, restriction fragments containing repetitive sequences were identified by hybridization to ^{32}P-labelled rat liver DNA and to clone pRB1 containing a rat Alu sequence equivalent (data not shown). Some of these repetitive sequences map inside the TO gene (Figure 3B).

Determination of the start sites of transcription of the TO gene

Because analysis of the steroid-regulated expression of the TO gene requires knowledge of the start site of the transcriptional unit, the DNA sequence around the 5′ end of the TO gene was first determined. Sequencing was carried out using the technique of Maxam and Gilbert (1980) as indicated in Figure 5B. The resulting nucleotide sequence is presented in Figure 5A.

The start site of transcription of the TO gene was determin-

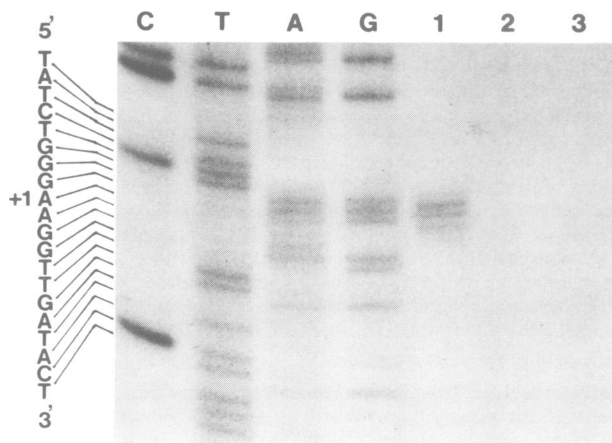


Fig. 7. Localization of the major TO mRNA start site by S1 nuclease mapping. **Lanes C, T, A,** and **G:** sequencing ladder from the *Hga*I 5′ end (+59 in Figure 5A). Note that the labelling of the sequencing lanes is transposed so that the mRNA sequence can be read directly. **Lane 1:** S1 nuclease-resistant products obtained by digesting a hybrid between rat liver total poly(A)$^+$ RNA and the *Hind*III-*Hga*I fragment (recognition sites at −292 and +45, Figure 5A), ^{32}P-labelled at the *Hga*I 5′ end at position +59. **Lane 2:** same as in **lane 1**, except that yeast tRNA was incubated with the probe. **Lane 3:** same as in **lane 1**, except that no RNA was added.

ed by S1 nuclease mapping (Weaver and Weissman, 1979). Total poly(A)$^+$ RNA from livers of dexamethasone-stimulated rats was hybridized to the *Hind*III-*Hga*I fragment (recognition sequences at −292 and +45, Figure 5A), 5′-labelled at the *Hga*I cleavage site at position +59. Fragments protected from digestion by S1 nuclease were sized on an 8% polyacrylamide sequencing gel. As shown in lane 1 of Figure 6A, two protected fragments are observed: a prominent fragment or series of fragments ~60 nucleotides in length (large arrow), and a much less prominent fragment of ~240 bases (small arrow). These two fragments could indicate two separate start sites for TO gene transcription. Alternatively, the faint band observed could be due to precursor mRNA protection of our probe, while the strong band could result from mature mRNA protection after processing of this precursor.

To decide between these two alternatives, an S1 protection experiment was conducted with a fragment probe terminating within the first intron which can be protected only by mRNA precursors. As intron probe we used a 465-bp *Hind*III-*Hind*II fragment (−292 to +173, Figure 5A), 5′-labelled at the *Hind*II site. As shown in lane 1 of Figure 6B, a fragment of ~175 nucleotides in length is observed, which maps to the same position as the major protected fragment seen in Figure 6A, lane 1. That the 5′ end of the TO nuclear RNA species detected by the intron probe maps to the same position as the 5′ end of the prominent TO mRNA species revealed by the exon probe supports the idea of two independent start sites for TO gene transcription: a major start site at position +1 (see also below), and a minor start site occurring ~180 bp further upstream (Figure 5A). Both start sites are in fact used in an *in vitro* transcription system (S.Hashimoto, unpublished data).

To determine more precisely the initiation point at the major start site, the experiment of Figure 6A was repeated and the sizes of the S1-resistant fragments were analyzed by comparison with a sequence ladder of the same restriction fragment. The results, shown in lane 1 of Figure 7, indicate that the two most prominent DNA fragments generated by S1 nuclease align with the GA residues in the sequence 5′-GG<u>GA</u>A-3′, if we take into account that S1-generated fragments are retarded by 1.5 nucleotides relative to chemically cleaved DNA on a sequencing gel (Tomizawa *et al.*, 1977). Since almost all eukaryotic mRNAs analyzed so far initiate with an A residue (Breathnach and Chambon, 1981), we assume that the start of the major TO mRNA is the A residue labelled +1 in Figure 5A and 7.

Discussion

We have identified a plasmid clone (pcTO-1) that contains rat TO cDNA sequences. As no amino acid sequence data are available for TO, matching nucleotide sequence with protein sequence is not possible. On the basis of the following two criteria we believe, however, that clone pcTO-1 contains authentic TO cDNA sequences. First, it selects a mRNA translating into a protein of the correct mol. wt. which is precipitated by TO antiserum (Figure 1). The specificity of this antiserum has been assessed by its complete inhibition of TO enzyme activity and by Ouchterlony double-diffusion as well as countercurrent electrophoresis yielding a single precipitin line (Röwekamp *et al.*, 1976; and unpublished data). Second, it hybridizes to a mRNA species which is inducible by dexamethasone (Figure 2). These conclusions are corroborated by our observation that TO mRNA is not detectable in the Fao cell line of rat hepatoma by probing with pcTO-1 (U.Danesch, unpublished data); all rat hepatomas and hepatoma cell lines analyzed so far lack TO enzyme activity as well as translatable TO mRNA (Ramanarayanan-Murthy *et al.*, 1976; Hofer *et al.*, 1978). In addition, analysis of mRNA after hormone stimulation, using clone pcTO-1 as probe, confirms and extends previous, more indirect measurements on the kinetics of TO induction (Danesch *et al.*, in preparation).

The 1900 bases [minus poly(A)] TO mRNA is encoded by a structural gene which is larger by a factor of 10 extending over 19 kb and which is interrupted by 11 introns. We cannot completely rule out the possibility that the TO gene extends beyond the cloned region shown in Figure 3. This is, however, very unlikely since the total length of the twelve exons (1875 ± 98 bases) corresponds closely to the measured size of the TO mRNA.

Because the restriction maps of the nine independently isolated genome clones overlap to form a unique map, we conclude that the rat TO gene is a single copy gene. This notion is supported by genomic blots probed with TO cDNA and genomic clones where only restriction fragment sizes expected from the physical map shown in Figure 3 are revealed (unpublished data). However, we do find repetitive sequences within some introns of the TO structural gene. Similar findings are now reported for a number of other well-characterized genes (Favera *et al.*, 1981; Page *et al.*, 1981; Tsukada *et al.*, 1982).

A region of 766 bases of genomic DNA around the 5′ end of the TO gene has been sequenced. Two start sites of transcription of the TO mRNA in that region have been detected by S1 nuclease mapping experiments: a major start site defining position +1 in Figure 5A, and a minor start site ~180 nucleotides further upstream (Figures 5A and 6A). Inspection of the nucleotide sequence reveals that the sequence CATAAAA, a variant of the consensus TATAAAA sequence characteristic of an RNA polymerase II promoter site

(Breathnach and Chambon, 1981), is located between nucleotides − 23 and −29 with respect to the major start site. Furthermore, between nucleotides −72 and −80 is a sequence resembling the 'CAAT'-box sequence common to a number of eukaryotic genes (Benoist *et al.*, 1980).

A second, perfect TATA-box sequence is found further upstream (position −203 to −209), at an appropriate distance from the minor start site of transcription (Figure 5A). While *in vivo* transcripts from this TATA promoter are by a factor of ~50 less abundant than transcripts starting from the CATA-promoter, both promoters function with about the same efficiency in an *in vitro* transcription system (S.Hashimoto, unpublished data). The low *in vivo* abundance of TO mRNA transcripts starting from the minor site probably explains why we do not detect these transcripts in the Northern blot experiment and in the S1 mapping experiment with the intron probe. Multiple start sites for RNA polymerase II transcription units have also been described for other cellular genes (Grez *et al.*, 1981; Hagenbüchle *et al.*, 1981).

The 3′ end of the first exon at position +93 (Figure 5A) was mapped by S1 protection experiments (not shown). The DNA sequence around this site shows excellent homology to the splice donor consensus sequence derived by Breathnach and Chambon (1981). Within the first exon we find an ATG that is followed by an open reading frame up to the potential splice site at +93. Although we have no experimental evidence, it is likely that translation initiates at this ATG codon.

We have used computer analysis to compare the 766 nucleotide TO gene sequence shown in Figure 5A with the first 220 nucleotides upstream of the start site of transcription in the long terminal repeat (LTR) of mouse mammary tumour virus (MMTV) (Donehower *et al.*, 1981; Fasel *et al.*, 1982; Kennedy *et al.*, 1982). It has been shown by gene transfer experiments and by DNA binding studies with glucocorticoid-receptor protein (GRP) that specific DNA sequences necessary for the dexamethasone stimulation of MMTV LTR gene expression and the GRP binding reside in the first 200 nucleotides upstream of the mRNA cap site (Majors and Varmus, cited in Cochet *et al.*, 1982; Govindan *et al.*, 1982; Govindan, personal communicatin). We find the sequence 5′-TCAGTTCTGA-3′ at position −101 to −110 in the TO gene (underlined in Figure 5A) which has 7 or 8/10 nucleotides in common with a similar sequence present at position −102 to −111 in the published MMTV LTR as indicated:

T G A G C T C T G A	(Donehower *et al.*, 1981)
T A A G C T C T G A	(Kennedy *et al.*, 1982)
T C A G T T C T G A	TO
T G A G C T C T T A	(Fasel *et al.*, 1982).

Whether these sequences actually are control signals involved in the glucocorticoid response of these genes has yet to be shown by functional tests.

The availability of TO cDNA and genomic clones − in conjunction with the TAT gene recently isolated in our lab (Scherer *et al.*, in preparation) − will allow us to study the molecular mechanism involved in the developmental and hormonal control of these two rat genes.

Materials and methods

Isolation and translation of RNA

Male Wistar rats (150 g) were injected i.p. with dexamethasone (Sigma) 10 μg/100 g body weight) 5 days after adrenalectomy. Control rats were injected with 0.9% NaCl. 4 h after injection, polysomal poly(A)$^+$ RNA was isolated as described by Röwekamp *et al.* (1976). Alternatively, total poly(A)$^+$ RNA was prepared according to LeMeur *et al.* (1981). To enrich for TO mRNA, 4 mg of dexamethasone-induced polysomal poly(A)$^+$ RNA were fractionated on a Sepharose 4B column (Pharmacia) in 0.1 M sodium acetate, pH 6.0, 1 mM EDTA. Fractions were assayed by translation and immunoprecipitation; those fractions containing TO mRNA were pooled and further fractionated by chromatography on Biogel A-15 (Biorad) in 20 mM sodium borate, pH 8.0, 5 mM methylmercury hydroxide. The final TO mRNA fractions (250 μg RNA) were pooled and the RNA was collected by ethanol precipitation. The translation assays were performed with a rabbit reticulocyte lysate by following the supplier's directions (New England Nuclear). Translation products and immunoprecipitates were analyzed by gel electrophoresis (Laemmli, 1970) and revealed by fluorography (Bonner and Laskey, 1974).

Construction of recombinant plasmids

Double-stranded cDNA was synthesized from the enriched TO mRNA with reverse transcriptase (a gift of J.Beard) as described (Röwekamp and Firtel, 1980). cDNAs and *Pst*I-cleaved pBR322 were tailed with dCTP and dGTP, respectively, by using terminal transferase (P.L.Biochemicals); hybrid molecules were constructed and used to transform competent *Escherichia coli* SF8. Transformants containing recombinant plasmids were selected for their resistance to tetracycline and sensitivity to ampicillin. Plasmid DNAs were purified by CsCl density gradient centrifugations in the presence of ethidium bromide. All experiments were performed under the containment conditions specified by the Zentrale Kommission für Biologische Sicherheit of the Federal Republic of Germany.

Hybridization-selected translation assay

10−20 μg of plasmid DNA were denatured and nicked by boiling in 0.1 N NaOH for 90 s, neutralized by addition of an equal volume of 2 M ammonium acetate, and adsorbed to nitrocellulose filters (BA 85, Schleicher and Schüll). Filters were baked at 70°C for 2−12 h *in vacuo*. Prehybridization was performed at 37°C for 24 h in 55% formamide, 30 mM Hepes, pH 6.7, 0.6 M NaCl, 2 mM EDTA, 0.1% SDS, after which the filters were boiled in water for 3 min. Extensive prehybridization was found to be essential for a strong hybrid selection since up to 90% of the fixed DNA may be lost during that step. Hybridization was carried out for 16 h at 37°C under continuous agitation in 200 μl of the same buffer containing 100−150 μg rat liver poly(A)$^+$ RNA isolated from dexamethasone-stimulated animals. After hybridization, the filters were washed as described (Ricciardi *et al.*, 1979). Hybridized mRNAs were recovered by boiling the filters three times in 200 μl each of water for 90 s, and precipitated in 80% ethanol, 0.4 M LiCl in the presence of 70% ethanol, dried, dissolved in water, and assayed by *in vitro* translation and immunoprecipitation (see above).

Screening of the rat genomic library

The bacteriophage λ Charon 4A library containing partial *Eco*RI fragments of rat liver DNA (Sargent *et al.*, 1979) was screened (Benton and Davis, 1977) by using nick-translated (Rigby *et al.*, 1977) pcTO-1 DNA as hybridization probe. Positive plaques were purified (three cycles) and DNA from phages was prepared according to Blattner *et al.* (1978).

Electrophoresis of RNA and blot-hybridization

Poly(A)$^+$ RNA was separated on 1% agarose formaldehyde gels and transferred to a nitrocellulose filter as described by Dobner *et al.* (1981). The blots were hybridized with ^{32}P-labelled DNA probes, washed, dried and subjected to autoradiography at −70°C with Kodak XAR-5 film, using intensifying screens.

Nucleotide sequence analysis

For sequence analysis, DNA fragments were labelled at their 5′ or 3′ ends and their sequence was determined as described by Maxam and Gilbert (1980). The *Bam*HI-*Pst*I fragment containing the 5′ end of the TO gene was subcloned into pUC8 (obtained by J.Messing) prior to sequence determination.

Heteroduplex analysis

λ recombinant DNAs were denatured in 0.2 N NaOH, loaded on a 1% native agarose gel and the separated single strands were adsorbed to, and subsequently eluted from, malachite green resin (Boehringer-Mannheim) as described by Koller *et al.* (1978). Heteroduplexes between the isolated DNA single strands (at 1−3 μg/ml) and enriched TO mRNA (at 10 μg/ml) were formed in 70% formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.5, 1 mM EDTA. Samples were kept at 54°C for 3 h, and then cooled down to room temperature over a period of several hours. The hybrids were prepared for electron microscopy as described (Herrmann *et al.*, 1978). To serve as internal length standard, and to orient the RNA-DNA heteroduplex regions relative to the λ vector arms, single strands of the Charon 4A recombinant λlys 30 (Lindenmaier *et al.*, 1979) were included in the hybridizations to form hybrid molecules with the λ TO DNAs *via* the common vector arms.

S1 mapping

S1 nuclease analysis was performed according to the procedure of Berk and Sharp (1977) as modified by Weaver and Weissmann (1979). The *Bam*HI-*Pst*I genomic subclone was digested with either *Hga*I or *Hind*II, treated with calf intestine phosphatase and 5′ end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. After recutting with *Hind*III, the 347-bp *Hga*I-*Hind*III subfragment or the 465-bp *Hind*II-*Hind*III subfragment were isolated, uniquely labelled either at the *Hga*I 5′ end at position +59 or at the *Hind*II 5′ end at position +175 (Figure 5A). Approximately 0.03 pmol of 5′ end-labelled DNA fragment (specific activity 1 x 10^6 c.p.m./pmol 5′ end) was mixed with 2 μg of total poy(A)$^+$ RNA isolated from dexamethasone-stimulated rats in 20 μl of 80% formamide, 400 mM NaCl, 40 mM Pipes, pH 6.4, 1 mM EDTA, heated to 85°C for 5 min and incubated overnight at 45°C. After addition of 10 volumes of S1 buffer (30 mM sodium acetate, pH 4.5, 3 mM $ZnSO_4$, 300 mM NaCl, 10 μg/ml denatured salmon sperm DNA) the hybrids were digested with S1 nuclease at a final concentration of 1000 units/ml for 3 h at 15°C. The reaction was terminated by phenol-chloroform extraction and the nuclease-resistant products were analyzed on 8% sequencing gels. In control experiments, rat liver RNA was substituted by 0.5 μg of yeast tRNA.

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