

**REGULATION of ORNITHINE- $\delta$ -AMINOTRANSFERASE in RETINOBLASTOMAS**

by

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## ABSTRACT

Ornithine Aminotransferase (OAT) is expressed at high levels in liver, kidney, and retina. Tissue-specific regulation of OAT has been described for rat kidney and liver. To characterize OAT regulation in retinal lines, we studied OAT synthesis in retinoblastomas RB355 and Y79.

OAT transcription and mRNA levels in the two lines were similar, but 3-fold greater immunoreactive OAT protein and enzyme activity were observed for Y79. Characterization of polysome-associated OAT mRNAs indicated that they were translated less efficiently, due to decreased initiation, in RB355. Initiation factor eIF-4E mRNA and protein were reduced in RB355; eIF-4E overexpression in RB355 increased OAT translation and OAT protein to the level observed in Y79.

Estrogen and thyroid hormone increased OAT expression in both strains. Estrogen increased translational initiation, with no effect on transcription, whereas thyroid hormone was primarily a transcriptional activator.

An alternatively-spliced OAT mRNA was identified in these retinoblastomas; this mRNA was poorly translated and was not affected by eIF-4E overexpression, estrogen, or thyroid hormone.

This study has demonstrated several post-transcriptional regulatory mechanisms for OAT in retinal tissue.

## SOMMAIRE

L'activité ornithine aminotransferase (OAT) est exprimée à de hauts niveaux dans le foie, les reins et la rétine. La régulation tissu-spécifique de cette enzyme a déjà été étudiée pour ce qui est du foie et des reins de rat. Dans le but d'étudier la régulation de l'activité OAT au niveau de la rétine, nous avons utilisé le système-modèle des cellules de rétinoblastome RB355 et Y79.

Les niveaux de transcription de l'ARNm d'OAT et d'accumulation du messager sont identiques entre ces deux lignées cellulaires mais la lignée Y79 possède trois fois plus de protéines OAT (détectées immunologiquement) et d'activité enzymatique correspondante. L'analyse des niveaux d'ARNm OAT associés aux polysomes a révélé que, chez la lignée cellulaire RB355, le messager OAT était traduit moins efficacement et ce, à cause d'un taux d'initiation diminué. Les niveaux de messagers et de protéines eIF-4E sont réduits chez la lignée RB355; une surexpression de eIF-4E dans la lignée RB355 a pour effet d'augmenter les niveaux de traduction de l'OAT et de la protéine correspondante les rendant comparables à ceux de la lignée Y79.

L'oestrogène et l'hormone thyroïdienne augmente la synthèse d'OAT dans les deux lignées cellulaires étudiées. L'oestrogène augmente l'initiation de la traduction du messager sans avoir aucun effet sur sa transcription tandis que l'hormone thyroïdienne est essentiellement un activateur transcriptionnel.

Un messenger d'OAT issu d'un épissage alternatif a été détecté dans ces lignées cellulaires de rétinoblastome. Cet ARN n'est pas traduit très efficacement et cette observation n'est en rien modifiée par une surexpression de eIF-4E ou d'un traitement à l'oestrogène ou à l'hormone thyroïdienne.

Cette étude a démontré qu'il existait plusieurs mécanismes de régulation post-transcriptionnelle pour l'ornithine aminotransferase quant à son expression au niveau de la rétine.

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Lastly, I co-dedicate this thesis to my parents and to Alyson. Mom and Dad, you made me what I am through your unfailing love and support. I think of you every day and miss you dearly. Al, I love you. The thought of a life spent alongside you excites me (as long as no unilateral decisions are made). You are the one for me today and always.

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## I. INTRODUCTION

### A. Ornithine- $\delta$ -Aminotransferase (OAT)

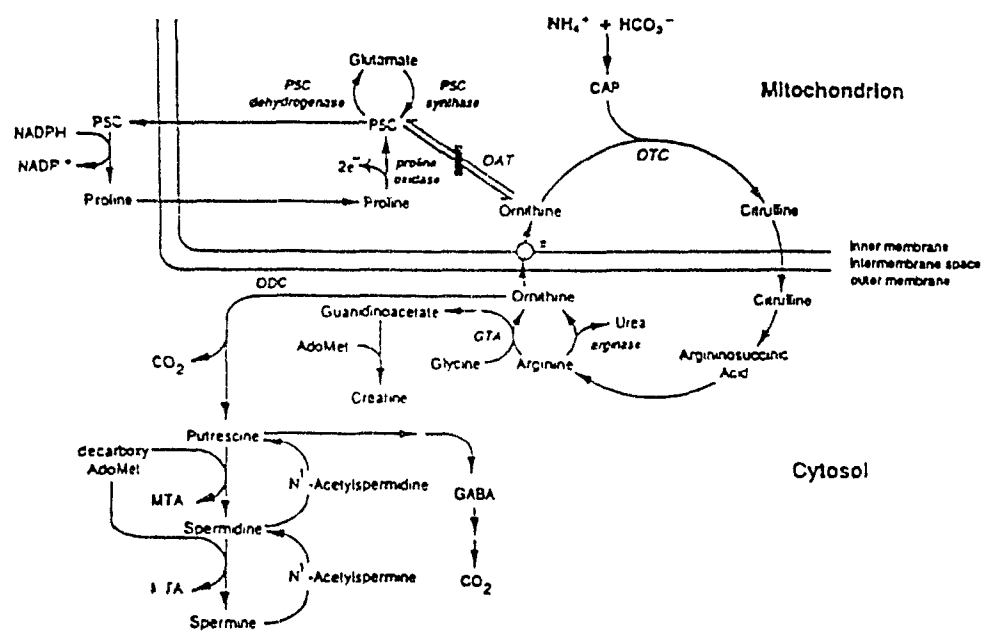
#### A.1 OAT Biochemistry

Ornithine is a non-protein amino acid which is directly involved in six different enzymatic reactions in the cell (figure 1). In the cytoplasm, ornithine decarboxylase (EC4.1.1.17) and glycine transamidase (EC2.1.4.1) convert ornithine to polyamines and creatine respectively. Ornithine is also the ligand for a specific transporter which carries it into the mitochondrial matrix (Valle et al 1989). In the mitochondrial matrix, ornithine is involved in several different metabolic reactions. It is a urea cycle intermediate; ornithine can be converted to citrulline by ornithine transcarbamylase (EC2.1.3.3). Citrulline is transported to the cytoplasm, and after several intermediates, is converted back to ornithine and urea, via the breakdown of arginine by arginase (EC3.5.3.1). Mitochondrial ornithine, instead of participating in the urea cycle, may also be metabolized to  $\Delta^1$ -pyrroline-5-carboxylate (P5C) by ornithine- $\delta$ -aminotransferase (OAT (EC2.6.1.13)). P5C can then be further metabolized to proline or glutamate. Glutamate may then be shunted into the tricarboxylic acid cycle, whereas

**Figure 1. Ornithine Metabolic Pathways.**

From Valle et al (1989). AdoMet=S-adenosylmethionine;  
CAP=carbamoyl phosphate; GABA= $\gamma$ -aminobutyric acid; GTA=glycine  
transamidinase; MTA=methylthioadenosine

fig.1



proline is required for collagen synthesis.

The main source of ornithine is dietary arginine, but under certain conditions, such as arginine deprivation, the OAT reaction may be reversed in order to synthesize ornithine. This reversal allows sufficient production of ornithine for the urea cycle (Valle et al 1989) and is the only de novo source of ornithine available to mammals (Valle et al 1989). The OAT reaction is therefore an important one, since it can regulate the availability of carbon atoms, in the form of ornithine, for the urea cycle, proline biosynthesis, and tricarboxylic acid cycle intermediates.

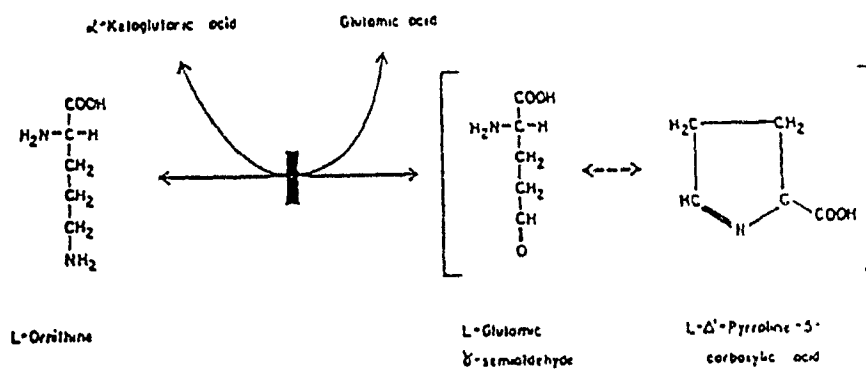
More specifically, OAT catalyzes the reversible transamination of ornithine and  $\alpha$ -ketoglutarate to glutamic semialdehyde and glutamate (figure 2). The glutamic semialdehyde is in equilibrium with its ring structure  $\Delta^1$ -pyrroline-5-carboxylate. The OAT reaction requires the cofactor pyridoxal-phosphate (Peraino et al 1983).

OAT has been purified to homogeneity from human liver (Ohura et al 1982), rat liver (Peraino et al 1969, Sanada et al 1970), and rat kidney (Sanada et al 1970, Kalita et al 1976). The native enzyme exists as either a homotetramer (Sanada et al 1976) or a homohexamer (Markovic-Housley et al 1987) depending on the NaCl concentration used during purification. Each subunit, in man, has an apparent molecular weight of 45 kilodaltons (kDa). It is a nuclear-encoded

Figure 2. Ornithine aminotransferase reaction.

From Valle et al (1989).

Ornithine + pyridoxal-ornithine- $\delta$ -aminotransferase  $\rightarrow$   
 $\Delta^1$ -pyrroline-5-carboxylate +  
 pyridoxamine-ornithine- $\delta$ -aminotransferase  
 Pyridoxamine-ornithine- $\delta$ -aminotransferase +  
 $\alpha$ -ketoglutarate  $\rightarrow$   
 glutamate + pyridoxal-ornithine- $\delta$ -aminotransferase



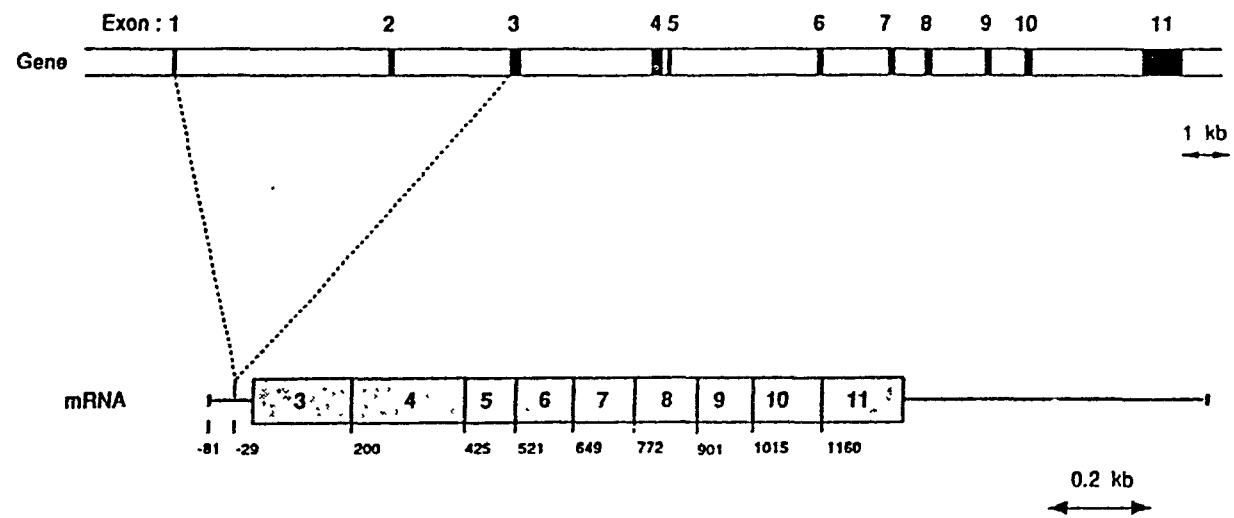
enzyme in which the subunits are synthesized on cytosolic free polysomes as 48,534 Da precursors (Mueckler et al 1982, Valle et al 1989). Upon mitochondrial entry, the mitochondrial leader peptide is cleaved between positions Ala-25 and Thr-26 (Simmaco et al 1986), leaving a 45,852 Da peptide.

## A.2 OAT Gene Structure and cDNA Analysis

OAT cDNAs have been isolated from human liver (Ramesh et al 1986, Mitchell et al 1988), human retinoblastomas (Inana et al 1986), rat liver (Mueckler et al 1985, Mitchell et al 1988) and *Saccharomyces cerevisiae* (Degols et al 1987) libraries. Mitchell et al. used a human liver cDNA to isolate the human OAT structural gene (Mitchell et al 1988). The functional OAT gene has been mapped to 10q26, while several OAT pseudogenes have been located at Xp11-Xp21. The OAT gene contains 11 exons, which span 21 kilobases (kb) of genomic sequence (Mitchell et al 1988) (figure 3). Two transcriptional start sites, one base pair (bp) apart, were predicted by primer extension analysis and RNase A analysis. The 5' most start site was assumed to be the conventional start site (Mitchell et al 1988). cDNA analysis predicted a mRNA which contained a 81 bp 5' untranslated region (UTR), 1317 bp of coding sequence, and a 635 bp 3'UTR (Mueckler et al 1985, Inana et al 1986, Mitchell et al 1988).

**Figure 3.** Onithine aminotransferase structural gene and mRNA.

From Valle et al (1989).



Comparison of the cloned OAT cDNA sequences to two OAT processed pseudogenes lead to the conclusion that exon 2, 87 nucleotides in length, was spliced out of the mature OAT message (Mitchell et al 1988) (figure 3). Inclusion of exon 2 in the mature message would create a 168 bp 5'UTR. Translation initiates 29 bp into exon 3 at a conventional translational initiation codon (Mitchell et al 1988). A single polyadenylation signal exists at nucleotides 1939-1944.

The human liver cDNA and rat liver cDNA show 84.3% and 90.4% homology at the nucleotide level and amino acid level respectively (Mitchell et al 1988). Similar analyses with the *Saccharomyces cerevisiae* OAT cDNA (Degols et al 1987) reveal 54.4% identity at the amino acid level. The areas of identity between the yeast and human OAT are scattered, but there are some areas of complete identity of up to 13 amino acids in length. These are areas of presumed functional importance. One such area encompasses the residue Lys-292. This is the site predicted by Simmaco et al (1986) to be the cofactor pyridoxal phosphate binding site.

### A.3 OAT Deficiency

Hereditary deficiency of OAT results in chorioretinal degeneration and hyperornithinemia, an autosomal recessive condition termed Gyrate Atrophy (Valle et al 1989). In most

patients, OAT activity from fibroblast extracts is undetectable or less than 5% of normal levels. Approximately 50% of normal OAT activity is observed in heterozygous carriers (Trijbels et al 1977, Valle et al 1977, Valle et al 1989).

Pyridoxal phosphate, a cofactor of OAT, administered in pharmacological doses as pyridoxine or vitamin B-6, alleviates hyperornithinemia in some gyrate atrophy patients (Shih et al 1978, Kennaway et al 1980). Consequently, gyrate atrophy has been subdivided into two classes: B-6 responsive and B-6 nonresponsive variants (Valle et al 1989). There are over 100 reported cases of gyrate atrophy, 50% of which are Finnish. Although the elucidation of the gene structure and sequence of OAT is a recent event, there already exists a substantial literature detailing the molecular defects associated with gyrate atrophy. The variety of defects is indicative of marked heterogeneity in the mutations at the OAT locus (Valle et al 1989). Although OAT is expressed in most tissues, the primary abnormality in OAT deficiency is retinal degeneration. The exact role of OAT in retinal tissue and the pathogenetic mechanisms are not well-defined.

#### A.4 Regulation of OAT

OAT is present in most tissues and is described as a "housekeeping" enzyme (Valle et al 1989). However, unlike

most enzymes of this class, OAT is highly subject to regulation in a tissue specific manner. OAT activity in rat is highest in liver, kidney, and small intestine (Hertzfeld et al 1968, Hertzfeld et al 1976A, Hertzfeld et al 1976B); the levels are modulated by a variety of stimuli. A comparative study of OAT levels from chick embryo, ox, and rat (Ratzlaff et al 1987), in retinal pigment epithelium, neural retina, and liver showed that OAT activity is highest in the retinal pigment epithelium. Little work studying OAT regulation in human tissues has been done.

In the most extensively studied tissues, rat liver and kidney, at least two classes of regulatory phenomena underlie changes of OAT activity: changes in the amount of OAT mRNA, and changes in the efficiency of translation of the mRNA. In rat kidney, estrogen and thyroid hormone induce OAT synthesis by increasing levels of its mRNA 13-fold and 2-fold (Mueckler et al 1983A, Mueckler et al 1984) respectively. When the two hormones are added simultaneously, a synergistic effect occurs, resulting in a 21-fold increase in OAT mRNA and activity (Mueckler et al 1983A). The increases in OAT mRNA account for the increases observed in OAT immunoprecipitable protein and activity. The rate of OAT mRNA synthesis, measured in rat kidney by nuclear run-on analysis after treatment with estrogen, increased only 3 to 6 fold (Mueckler et al 1984). A similar analysis with thyroid hormone showed a 2-fold increase in the rate of OAT mRNA synthesis (Mueckler

et al 1984). Therefore thyroid hormone appears to exert its effects at the transcriptional level, whereas estrogen may affect OAT synthesis at both the transcriptional and post-transcriptional levels. Interestingly, the time course for the response to these hormones differed, indicating a possible different mode of action.

Modulators of hepatic OAT include a high protein diet, glucagon, and dibutyryl cAMP (Peraino et al 1964, Sanada et al 1970). Glucose is able to repress the induction observed after a high protein diet (Peraino et al 1964).

When rats were maintained on a 60% protein diet, relative to a 0% diet, the rate of OAT synthesis increased up to 100-fold (Mueckler et al 1983B). Glucose repressed these increases (Peraino et al 1964). Glucagon administration to whole animals induced OAT enzyme synthesis by 10-fold (Mueckler et al 1983B), whereas dibutyryl cAMP had a 8-fold stimulatory effect on OAT synthesis in cultured hepatocytes (Merrill et al 1985A, Merrill et al 1985B). The effects of a high protein diet and glucagon administration are presumably mediated through cAMP. Measurement of OAT mRNA levels in rat liver (Mueckler et al 1983B) and cultured hepatocytes (Merrill et al 1985A) after administration of a high protein diet or cAMP, respectively, revealed that the increase in mRNA levels, 2 and 3 fold respectively, only accounted for a fraction of the increase observed in OAT synthesis. Further investigation revealed that cAMP increased the rate of initiation of

translation of the OAT message by approximately 3-fold in cultured hepatocytes (Merrill et al 1985A). When hepatic OAT was examined from whole animals maintained on a high protein diet, the rate of initiation of translation was found to be increased 23-fold, while the rate of elongation was increased 2-fold (Mueckler et al 1983B). These rates were established by polysome analysis in the presence of cycloheximide (an inhibitor of elongation) and by direct measurement of the ribosomal transit times on the OAT message.

The above-mentioned effects are tissue-specific; modulators of hepatic OAT have no effect on kidney OAT, and vice versa (Sanada et al 1970, Lyons et al 1977). Therefore, depending on the tissue, OAT experiences regulation at both the transcriptional and translational levels.

## B. Transcriptional Regulation

### B.1 Transcription in Eucaryotes

In mammalian cells, transcription of mRNA by RNA polymerase II is mediated by ubiquitous general factors that act through core promoter elements, usually a TATA box and associated initiation site sequences (for review see Saltzman et al 1989, Sawadogo et al 1990). Initiation of mRNA synthesis is a primary control point in the regulation of differential gene expression. Regulation occurs by tissue-

specific factors that can act from distal sites, through varied sequences, on the responding gene's promoter (for review see Johnson et al 1989, Mitchell et al 1989). Transcriptional initiation/regulation has been a widely-studied and well-documented area of research. As such, a complete review is beyond the scope of this thesis and references for reviews are included where necessary. The following section will focus on transcriptional regulation by steroid and thyroid hormones.

## B.2 Steroid and Thyroid Hormones

Steroid and thyroid hormones are synthesized in response to a variety of neuroendocrine activities, and exert major effects on growth, tissue development, and cellular homeostasis via regulation of a variety of pathways and of intermediary metabolism. The major groups of steroids are the corticoids or adrenal steroids, the steroid sex hormones, and vitamin D. The corticoids include glucocorticoids and mineralocorticoids whereas the sex steroids include the estrogens and the androgens. These are all synthesized from cholesterol. Thyroid hormones are synthesized as iodinated forms of tyrosine.

These molecules are generally considered to exert their effects at the level of gene expression. After binding to specific receptors, the hormone receptor complex is able to activate or repress expression of certain genes in a tissue-

specific manner. Although steroid and thyroid hormones are generally considered to affect gene expression primarily by modulation of mRNA levels (Anderson et al 1985), reports on the post-transcriptional effects of these hormones are prevalent.

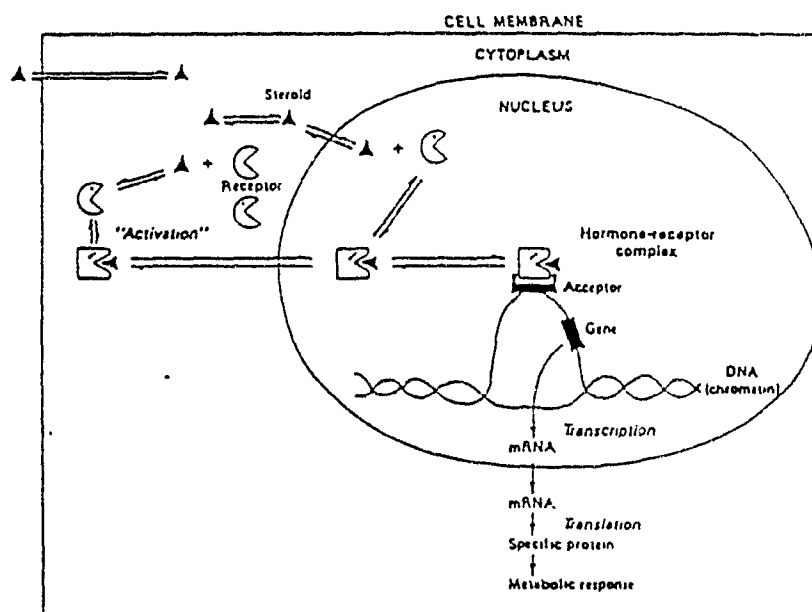
At the transcriptional level, these hormones work through the same mechanism, through binding to a specific receptor (figure 4). In an unbound form, the hormone receptor does not have a high affinity for DNA. The unbound receptor may be in equilibrium between the cytoplasm and the nucleus, but once the hormone binds the receptor, the complex is always found in the nucleus (Anderson et al 1985). Binding of the hormone to the receptor causes it to undergo activation or transformation. Once the receptor is activated, it attains a high affinity for DNA sequences. The complex mediates transcriptional activation via a specific sequence of DNA, the response element. These sequences are termed hormonal responsive elements (HREs), and a HREs exists for each hormone, eg. the estrogen responsive element. The HRE is usually located in the enhancer region of a hormonally-responsive gene. These sequences behave as enhancers in that they operate on a cis-linked promoter at great distances in an orientation-independent manner (Beato 1989). When the hormone receptor complex binds to the HRE, the promoter is activated, and transcription is initiated.

The *Xenopus vitellogenin* gene (Burch et al 1988), the rat

**Figure 4.** The mechanism of transcriptional activation by steroid hormones.

From Anderson et al (1985).

fig. 4



prolactin gene (Maurer et al 1982), and the human p52 gene (Masiakowski et al 1982) are some of the genes modulated by estrogen. The transcriptional response to estrogen is modulated through an ERE, GGTCANNNTGACC (Beato 1989). Thyroid hormone has been shown to be a transcriptional effector of rat growth hormone (Glass et al 1987), phosphoenolpyruvate carboxykinase (Hartong et al 1987), and malic enzyme (Dozin et al 1986). Its action is modulated through a TRE, TCAGGTCA---TGACCTGA (Hudson et al 1990).

Both the estrogen and thyroid hormone receptors are members of a superfamily of ligand-binding transcriptional activators (Evans 1988). The receptors in this family are modular; they have independent domains for hormone binding, DNA binding and transactivation (Eberwine et al 1984, Beato et al 1989). There is a centrally located region of 66 to 88 amino acids with nine conserved cysteine residues, which form zinc fingers. This area is essential for DNA binding and for specificity of target gene activation. The carboxy terminal part of the receptor is required for ligand binding and regulates transformation of the receptor to an active form. The amino terminus determines, in part, the efficiency and specificity of transactivation (Evans 1988, Beato 1989).

Steroid and thyroid hormones also repress genes at the transcriptional level. The pro-opiomelanocortin gene (Eberwine et al 1984) and the prolactin gene (Camper et al 1985) are transcriptionally repressed by the presence of

glucocorticoids. The activated glucocorticoid receptor binds to a consensus sequence very similar to that of a GRE. Thyroid hormone inhibits transcription of the  $\alpha$  and  $\beta$  thyrotropin genes (Catterjee et al 1989). It binds to the  $\alpha$  thyrotropin gene promoter immediately downstream of the TATA box and, in this way, inhibits initiation of transcription. Thyroid hormone and retinoic acid receptors mediate ligand-dependent inhibition of epidermal growth factor receptor promoter activity (Hudson et al 1990).

### B.3 Transcriptional Regulation of OAT

The transcriptional regulation of OAT is modulated through discrete elements in the promoter region of the OAT gene. Analysis by Englehardt et al (1991) of the 5' flanking sequences to the transcriptional start site revealed both standard and specific promoter/enhancer elements (figure 5). The transcription start site lies 3' to a TATA box, 3 CCAAT boxes, and 3 GC rich SP1 binding sites. SP1 elements are a common feature of "housekeeping" genes (Saltzman et al 1989, Sawadogo et al 1990) whereas the presence of a TATA box is not common, but is not unprecedented (Saltzman et al 1989, Sawadogo et al 1990). Two sequences with incomplete or complete dyad symmetry were found at -20 and -215 respectively (Engelhardt et al 1991). Areas of dyad symmetry are associated with regulatory protein-binding sites, as has been

**Figure 5.** The 5' flanking region of the human ornithine aminotransferase gene.

From Engelhardt et al (1991). UCE=urea cycle element; INV  
CAAT=inverted CAAT box

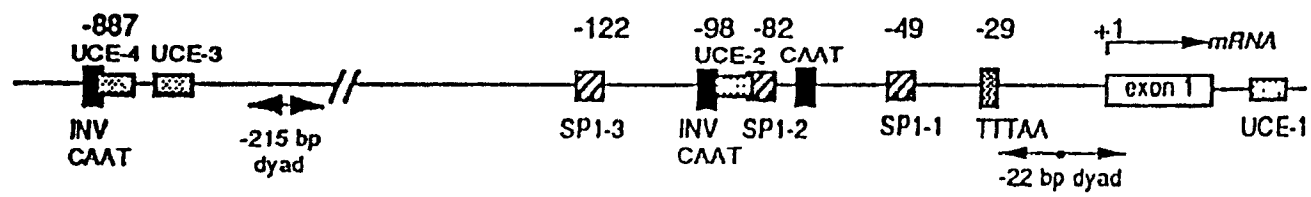


fig. 5

shown for the estrogen, cAMP, and glucocorticoid (Beato 1989) responsive elements. The incomplete dyad at -20 extends over the transcriptional start site to +2, and exhibits strong homology to both a cAMP responsive element and an estrogen responsive element. The element with complete symmetry at -215 does not have homology to any known regulatory elements.

When the 5' flanking sequences were fused to a reporter gene and tested for their ability to stimulate transcription, all of exon 1, which is part of the 5' UTR, had to be included in the constructs in order to obtain maximal basal promoter activity. This suggested that sequences in exon 1 are involved in gene regulation. cAMP elicited a 4-fold stimulation of this construct in HEPG2 cells, although no specific element was identified that conferred cAMP inducibility (Engelhardt et al 1991). The ability of estrogen or thyroid hormone to activate transcription was not tested.

Ohtake et al (1988) had identified a 13 bp element, in the promoters of several urea cycle enzymes, which was termed the urea cycle element (UCE). The function of this element is unknown. Sequences homologous to this element were found in four areas of the OAT 5' flanking sequences.

DNA footprint analysis of the OAT promoter region, using nuclear extracts from rat kidney and liver, showed tissue-specific patterns of protection over one UCE, UCE-2, and over the palindromic sequence GGGACCC, bp -104 to -110 (Engelhardt et al 1991). These sites may be binding sites for liver- or

kidney-specific factors that are involved in the tissue-specific transcriptional regulation of OAT.

### C. Translational Regulation

#### C.1 Translation in Eucaryotes

The process of protein translation can be divided into three phases: initiation, elongation, and termination. The initiation phase of protein synthesis involves three steps (figure 6): A) formation of the 43S preinitiation complex, B) formation of the 48S preinitiation complex, consisting of mRNA binding to the 43S complex, followed by scanning for the initiation codon, C) formation of the 80S initiation complex.

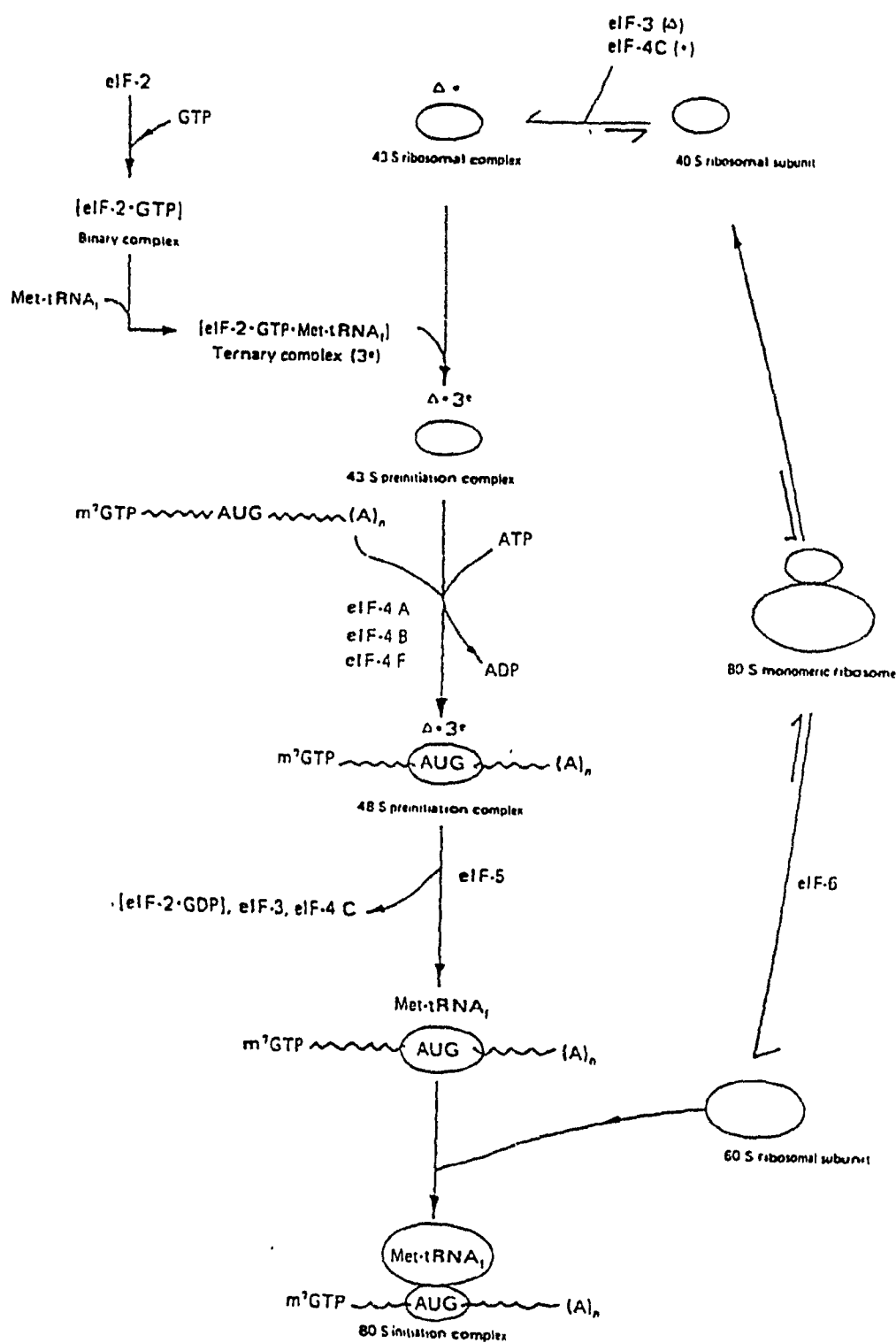
In part A, a binary complex between eIF-2 (eucaryotic initiation factor-2) and GTP is formed. Met-tRNA, the initiator tRNA, then binds to the complex to form a ternary complex. The ternary complex then binds to a 43S ribosomal complex, which consists of the 40S ribosomal subunit and the initiation factors eIF-3 and eIF-4C.

In the next stage, part B, the 43S preinitiation complex binds to the 5' end of a mRNA. In order for this to occur, the 5' end of the mRNA interacts with eIF-4A, eIF-4B, and eIF-4F. eIF-4F is a multicomponent complex consisting of eIF-4E, eIF-4A and a 220-kDa protein termed p220. Although it is not clear whether eIF-4A acts alone or as part of the eIF-4F

**Figure 6.** Initiation of protein synthesis in mammalian cells.

From Pain (1986).

fig. 6



complex (Rhoads et al 1988), all 3 factors (eIF-4A,B,F) are needed for optimal binding. It is eIF-4E, the 24-kDa protein component of eIF-4F, that is the first factor to interact with the mRNA. It specifically binds the methyl-7-guanosine cap of the mRNA in an ATP-independent reaction. The second factor to come into contact with the mRNA is probably eIF-4A. This factor most likely mediates ATP-dependent unwinding of mRNA secondary structure, and presumably generates a more suitable environment for binding of the initiating ribosomal subunit (Rozen et al 1990). The presence of eIF-4B enhances these reactions (Ray et al 1985). The 43S preinitiation complex then moves along the 5'UTR until it reaches the correct initiation codon; at this point it is referred to as the 48S preinitiation complex.

In the final stage of initiation, part C, eIF-5 promotes the release of the initiation factors bound to the 40S subunit. This requires the hydrolysis of the GTP molecule that entered as part of the ternary complex. A 60S subunit can then bind to the 40S•mRNA complex, forming an 80S initiation complex on the mRNA. The eIF-2•GDP can then be recycled to eIF-2•GTP by guanine exchange nucleotide factor, or eIF-2B, in order to start a new round of initiation.

The elongation steps of protein synthesis form a cycle. Aminoacyl-tRNA, in the form of a ternary complex with EF-Tu (elongation factor-Tu) and GTP, binds to the A site of the ribosome, followed by GTP hydrolysis and the ejection of EF-

Tu•GDP. Peptide bond formation occurs and the newly- formed peptidyl•tRNA•mRNA complex in the A site is translocated to the P site in a reaction catalyzed by EF-2 and GTP hydrolysis.

Termination of protein synthesis is promoted by eRF, eucaryotic release factor. eRF•GTP is needed for eRF to bind to ribosomes. The termination reaction involves release of the completed polypeptide from the last tRNA, release of the tRNA from the ribosome, and dissociation of the ribosome from the mRNA.

## C.2 Regulation of Translation

Regulation of protein synthesis occurs mainly at the level of polypeptide chain initiation, although there have been reports documenting translational regulation at the level of polypeptide chain elongation. In some instances, both processes are affected; the responses to heat shock (Ballinger et al 1983), and influenza virus or adenovirus infection (Katze et al 1986) involve a slow initiation rate with a slow elongation rate to discriminate against normal cellular protein synthesis.

Initiation is a logical point to exert control of translation, since it is the first step in the process. Regulation may occur by at least three mechanisms:

- 1) formation of ribonucleoprotein (RNP) particles - mRNAs may form complexes with proteins and/or small RNAs to

produce RNP particles. This process alters the affinity for the translational machinery and sequesters the mRNAs away from translational components. This type of regulation has been observed for many mRNAs in unfertilized sea urchin embryos (Jenkins et al 1978, Kaumeyer et al 1978), and for myosin heavy chain mRNAs prior to myogenesis (Dym et al 1979).

2) modification of translational components - A modification of the translational machinery may alter its affinity for a subset of mRNAs. Examples of this mechanism include the inhibition of host cellular protein synthesis during adenovirus (Reichel et al 1985, Schneider et al 1985) and polio virus (Etchison et al 1982, Etchison et al 1984) infection. This inhibition results from alterations of initiation factors eIF-2 (Siekierka et al 1985) and the p220 component of eIF-4F (Etchison et al 1982) respectively. Translational control through reversible phosphorylation (inhibition) and dephosphorylation (activation) of eIF-2 may represent a major mechanism to control translational initiation. Many conditions are known to affect the activity of eIF-2, including nutrient supply, hormonal stimulus, viral infection, and heat shock (Pain 1986). Two protein kinases have been characterized which phosphorylate the  $\alpha$  subunit of eIF-2. The first is heme controlled inhibitory kinase, HCI, which is found largely in reticulocytes (Hershey et al 1986). HCI is active as a kinase in the absence of heme, and specifically phosphorylates the  $\alpha$  subunit of eIF-2. This

results in formation of a phosphorylated eIF-2•eIF-2B (guanine exchange nucleotide factor) complex, that inhibits eIF-2B catalytic activity and consequently polypeptide chain initiation.

The second protein kinase is the double-stranded RNA activated protein kinase (DAI). DAI is present in most cell types and is activated by low concentrations of double-stranded RNA (Hershey et al 1986). The phosphorylation of eIF-2 by DAI results in inhibition of polypeptide chain initiation in a manner identical to that for HCI.

Duncan and Hershey (1984) demonstrated increased phosphorylation of eIF-2, in response to heat shock, albeit not through HCI or DAI; dephosphorylation of eIF-4B was also observed.

eIF-4E, the 24 kDa component of eIF-4F, is also subject to phosphorylation (Duncan et al 1987). In order to be functional as the cap binding protein, eIF-4E must be phosphorylated at serine residue 53 (Rychlik et al 1987). eIF-4E is present as a mixture of phosphorylated and non-phosphorylated forms in reticulocytes (Rychlik et al 1986), HeLa cells (Duncan et al 1987), and Swiss 3T3 fibroblasts (Kaspar et al 1990). Evidence exists that the phosphorylation state of eIF-4E correlates with the rate of protein synthesis. In HeLa cells, heat shock decreases both the phosphorylation of eIF-4E and the rate of protein synthesis initiation (Duncan et al 1987), and protein synthesis is restored by a complex of

p220 and highly phosphorylated eIF-4E (Lamphear et al 1990). There are simultaneous increases in the rate of phosphorylation of eIF-4E and protein synthesis in rabbit reticulolysates stimulated by phorbol esters (Morley et al 1989), in 3T3-LI fibroblasts treated with insulin (Morley et al 1990), in Swiss 3T3 fibroblasts stimulated with serum (Kaspar et al 1990), and in human cervical carcinoma cells treated with tumor necrosis factor  $\alpha$  (Marino et al 1989).

Polio virus infection of HeLa cells causes selective inhibition of host protein synthesis followed by exclusive synthesis of viral proteins (Etchison et al 1982). This is caused by proteolysis of p220, the 220 kDa protein component of eIF-4F. Analysis has shown that in the absence of functional p220, eIF-4F is unable to bind the mRNA cap structure (Sonenberg 1988). Therefore all capped mRNAs are rendered untranslatable.

3) mRNA structure - Some mRNAs may compete more efficiently for some rate-limiting components of the translational machinery. This has been observed with encephalomyocarditis virus mRNAs (Lawrence et al 1974, Jen et al 1978), and reovirus mRNAs (Brendler et al 1981). Kinetic studies have implicated 2 mRNA binding factors, eIF-4F and eIF-4A, as being capable of discriminating among mRNAs in in vitro translation systems (Lawson et al 1988). eIF-4F is the limiting component, in amount, among translation initiation factors (Sonenberg 1988). This is due to the low abundance of

eIF-4E (Duncan et al 1987), the limiting component of the eIF-4F complex.

eIF-4E is the initiation factor present in the least amount in the cell (Duncan et al 1987). Overexpression of eIF-4E in NIH 3T3 or Rat 2 fibroblasts (Lazaris-Karatzas et al 1990), and HeLa cells (De Benedetti et al 1990) resulted in malignant transformation of the transfectants. Therefore, it has been hypothesized that the limiting nature of active eIF-4E may be important in the translational regulation of growth-related mRNAs. In this regard, Kaspar et al (1990) showed that increased amounts of phosphorylated eIF-4E correlated with the translocation of the mRNA for ribosomal protein L32 from the mRNP pool into polysomes.

Data has indicated that the individual features of mRNAs that may be recognized by eIF-4F, and thus serve as a basis for discrimination, are mRNA secondary and tertiary structure. Godefroy-Coburn et al. (1985) have proposed that the high degree of translational regulation among the four alfalfa mosaic virus mRNAs was due to the steric accessibility of the cap structure. Pelletier et al. (1985) have proposed that 5' proximal secondary structure plays a role in modulating translational efficiency. They have shown that the mRNAs of certain viruses (Edery et al 1984) and reporter genes (Pelletier et al 1985) that have a high degree of secondary structure are translated less efficiently. They have hypothesized that initiation of these mRNAs is limited by

unwinding of 5' proximal secondary structure; therefore higher concentrations of initiation factors are required for optimal translation of these messages (Edery et al 1984).

Structures, such as hairpins and stem loops (Brawerman 1987, Atwater et al 1990), in the 5' and 3' UTR of mRNAs are able to impart stability to several mRNAs. Hairpins in concert with specific binding proteins, have been shown to regulate the stability of transferrin receptor mRNAs (Koeller et al 1989) as well as the translatability of ferritin mRNAs (Caughman et al 1988). Another characteristic of the 5'UTR of some translationally-regulated mRNAs is short open reading frames. A segment of the yeast GCN4 mRNA containing 4 AUG codons was able to confer translational control upon a heterologous yeast transcript (Mueller et al 1987).

The possibility that mRNA can be protected by ribosomes from endonucleolytic cleavage has also been investigated. Kelly et al. (1987) , using several developmentally-regulated mRNAs, showed that interaction of the ribosomes with the mRNAs played a role in the stability of the message. Belasco et al. (1986) investigated the half-life of the ompA transcript in *E. coli*; they concluded that the long half-life was, in part, conferred by ribosomal interaction with the 5'UTR. This mechanism may also be responsible for the rapid degradation of messages that contain nonsense mutations, such as mutagenized dihydrofolate reductase mRNAs (Urlaug et al 1989). As the ribosome encounters the stop codon, the rest of the message is

left naked, and may therefore be more susceptible to endonucleolytic cleavage. In support of this hypothesis, immunoglobulin mRNAs with nonsense mutations in the 5' end of the coding region are much less stable than those with the mutations near the authentic termination codon (Atwater et al 1987).

### C.3 Post-Transcriptional and Translational Regulation by Steroid Hormones

Steroid hormones have been reported to exert an effect at the translational level in several systems. For example, several steroid hormones have been shown to alter the rate of protein synthesis globally for the mRNAs of specific tissues. Pennequin et al. (1978) and Palmiter (1972) have reported that estrogen can alter the rate of initiation of protein synthesis of all mRNAs in chick oviduct. Estrogen increased the rate of peptide elongation on uterine ribosomes (Labate et al 1986), whereas androgens increased the rate of initiation of peptide synthesis on mRNAs of the ventral prostate of rats (Liand et al 1985).

Effects on translation of specific mRNAs have been demonstrated in vivo and in vitro. Progesterone selectively increased the rate of initiation of ovalbumin mRNA from chick oviduct (Pennequin et al 1978). Glucocorticoid treatment of lymphosarcoma cells resulted in a decrease in polysome loading

of ribosomal protein mRNAs (Meyuhas et al 1987).

A number of steroid hormones have been shown to increase the rate of translation of myelin basic proteins in a cell-free translation system (Verdi et al 1990). The translational regulation observed for the myelin basic proteins was shown to be governed by a discrete element in the 5' UTR of the myelin basic protein mRNA (Verdi et al 1990). The mechanism of action was not proven, but it was hypothesized that the steroids interacted with component(s) of the translational machinery and, simultaneously, with the identified element in the mRNA to enhance translational initiation.

mRNA stability has also been demonstrated to be affected by steroid hormones. Glucocorticoids alter the stability of mRNAs coding for interferon (Peppel et al 1991), phosphoenolpyruvate carboxykinase (Peterson et al 1989), and rat and human growth hormone (Birnbaum et al 1986, Paek et al 1987). Estrogen stabilizes ovalbumin mRNA in chick oviduct (Palmitter et al 1974) and vitellogenin mRNA in chick liver (Gordon et al 1988). The determinants of stability differ in most cases. For example, an AU rich sequence in the 3' UTR of the interferon mRNA may be responsible for the glucocorticoid-induced stability (Peppel et al 1991), whereas an increase in the poly A tail length of human growth hormone mRNA may relate to the increased stability (Paek et al 1987). An estrogen-dependent protein that binds the 5' UTR of the vitellogenin mRNA has been reported, but its involvement in estrogen-

mediated stabilization is not yet known (Liang et al 1991).

#### C.4 Post-Transcriptional and Translational Regulation of OAT

As previously discussed in section A.4, OAT can be regulated at the translational level, during initiation or elongation (Mueckler et al 1983B). Both initiation and elongation were increased in response to cAMP in rat liver, but the mechanisms were not identified. The increase in OAT activity after estrogen treatment (in rat kidney) was not fully-accounted for by increases in transcription (Mueckler et al 1984), suggesting that estrogen may also play a role in the post-transcriptional regulation of OAT.

## II. PROPOSAL

Ornithine Aminotransferase (OAT) is expressed to varying degrees in most tissues of rat; high levels of activity have been demonstrated for rat liver, kidney, and small intestine. Interestingly, the highest level, 3-10-fold that of liver, was reported for the retinal pigment epithelium of chick embryo, ox, and rat (Ratzlaff et al 1987).

A generalized deficiency of OAT in man results in gyrate atrophy of the choroid and retina, a disorder which is characterized by degeneration of the retina. Although this observation suggests that functional OAT is necessary for retinal well-being, the exact mechanism underlying the susceptibility of retinal cells to OAT deficiency is not known.

The tissue-specific regulation of OAT in rat liver and kidney has been relatively well-documented, yet little or no examination of OAT regulation has been performed in retinal tissue or in human tissues. Considering the high level of OAT activity in retina and the importance of OAT to retinal integrity, it is likely that complex regulatory mechanisms would exist for this tissue as well. Consequently, I have undertaken a study to characterize the regulation of OAT in the retinoblastomas RB355 and Y79.

Retinoblastomas were chosen as the cell line of study for two reasons: they are retinally-derived human tissue, and they

are easier to maintain in culture, as compared to primary retinal cultures. Two different strains will be examined to assess whether results are strain-specific, or are a general property of this tissue.

Synthesis of OAT protein and enzymatic assays for OAT will first be performed in the absence of any known modulators of OAT expression. Evaluation of the rate of gene transcription and of the efficiency of translation of the OAT mRNA will aid in establishing baseline OAT control mechanisms in the RB355 and Y79 strains.

Estrogen and thyroid hormone are known modulators of OAT synthesis. The effects of these two hormones on OAT synthesis will be examined in retinoblastomas. Transcriptional and post-transcriptional mechanisms of regulation will be investigated.

This study will provide the first description of the regulatory mechanisms for OAT in retinal cultures, as a model system for OAT expression in retina in vivo.

### **III. MATERIALS and METHODS**

#### **Purification of OAT and Production of Antiserum**

Rats were fed a high protein diet (60% casein) for 3 days prior to sacrifice. OAT was purified from rat liver as described by Peraino et al (1969). Preparations approximately 95% homogeneous (as judged by laser densitometry of Coomassie-stained SDS gels) and 1000-fold enriched for OAT activity were obtained, and employed to immunize rabbits. Four injections of 0.3 mg OAT in Freund's adjuvant were given over the course of six weeks; complete Freund's adjuvant for injections at days 0 and 7, and incomplete for those at days 21 and 42. Bloods were taken commencing 7 weeks after the initial injection. The antiserum reacted with a single major protein band (~45 kDa) on immunoblots of either rat liver mitochondria or a post-nuclear supernatant. One major band was immunoprecipitated from total rat liver mRNA translation products (~49kDa). The OAT purification and production of antiserum were performed by William Sheffield in the laboratory of Dr. Gordon Shore (Biochemistry Dept., McGill University).

#### **Cell Lines**

Retinoblastomas RB355 and Y79 were kindly provided by Dr. B. Gallie (Univ. of Toronto, Toronto, Ontario). They were maintained in culture in Dulbecco's Modified Eagle's Medium

(DMEM, Gibco), 15% fetal calf serum, 5 mg/L insulin, and  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol. For the hormonal experiments, after extensive washing with PBS, the cells were incubated in serum-free MEM with estradiol (Sigma) or  $T_3$  (Sigma) at a final concentration of  $10^{-6}$  M. After the indicated time of treatment, cell pellets were collected.

### Enzyme Activity

OAT activity was measured according to Ohura et al (1983). Briefly, cell pellets were sonicated in 50 mM potassium phosphate (pH 8.0), 5 mM  $\alpha$ -keto-glutarate, 20 mM pyridoxal phosphate (PLP) and centrifuged for 30 mins. at 105,000xg at 4°C. Supernatants (40  $\mu$ g and 80  $\mu$ g protein) were assayed in 250  $\mu$ ls of the reaction mix, consisting of 50 mM potassium phosphate (pH 8.0), 20 mM PLP, 10 mM  $\alpha$ -keto-glutarate, and 1 mg/ml bovine serum albumin. 7.5 $\mu$ l of [U- $^{14}$ C] ornithine (NEN, 266.0 Ci/mol) was added and the reaction continued for 30 minutes at 37° C. Radiolabelled pyrroline-5-carboxylate was reacted with o-aminobenzaldehyde (Aldrich), and then extracted with sodium sulfate and butanol. The rate of incorporation was determined by liquid scintillation counting. Protein concentrations were determined using the BioRad microassay procedure according to the instructions of the supplier (BioRad).

### Immunoblotting-OAT and Actin

Cell extracts were prepared by sonication of cell pellets in TBS (20mM Tris (pH 7.5), 500mM NaCl) and centrifugation for 30 mins. at 105,000xg at 4°C. Protein concentrations were determined as mentioned above. For dot blot analysis, 20, 40, and 80 µg protein of the supernatant fraction were dot-blotted to a nitrocellulose membrane using a BioRad manifold. For Western analysis, 80µg protein of the supernatant fraction was electrophoresed on a SDS-polyacrylamide gel, followed by transfer to a nitrocellulose membrane according to Sambrook et al. (1989) for 4 hours, 70V, at 4°C. In both cases, the membranes were blocked for 1 hour in 3% gelatin/TBS, and then probed with anti-OAT at 1/500 dilution in 1% gelatin/TBS. After washing in TBS, the membranes were incubated with alkaline phosphatase-linked goat anti-rabbit antibody, which was purchased from BioRad and used according to instructions of the supplier. Antiserum against actin, (BioRad), was used as an internal control. Immunoblots were scanned with a LKB densitometer; the results are presented as % area under normalized curve.

#### **Immunoblotting-eIF-4E and Actin**

Cell extracts were prepared by sonication of cell pellets in lysis buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 100µg/ml phenylmethylsulfonylfluoride (PMSF), 0.5% Nonidet P-40) and centrifugation for 20 mins at 100 000 xg at 4°C. 100µg protein from the supernatant fractions were electrophoresed on

SDS-polyacrylamide gels followed by transfer to a nitrocellulose membrane according to Sambrook et al (1989) for 4 hrs, 70V, at 4°C. The membranes were blocked with 5% non-fat dried skim milk powder in PBS for 2 hrs at room temperature and then incubated overnight at room temperature with a polyclonal rabbit antiserum against mouse eIF-4E synthetic peptide (Lazaris-Karatzas et al 1990) (kindly provided by Dr. N. Sonenberg, Biochemistry Dept., McGill University), at a dilution of 1:3000 in PBS. After washing in PBS, the membranes were incubated with alkaline phosphatase-linked goat anti-rabbit antibody (BioRad) according to the instructions of the supplier. Antiserum against actin was used as an internal control.

#### **cDNA Probes and Radiolabelling**

Lambda gt11 phage containing a human OAT cDNA was kindly provided by Dr. M. Mueckler (Washington University, St. Louis, Missouri). The insert (~1.8kb) was subcloned into the EcoRI site of the Bluescript vector (Promega) and partially sequenced to confirm its identity.

A 1.4 kb EcoRI/HindIII fragment of the murine eIF-4E cDNA (Lazaris-Karatzas et al 1990) was kindly provided by Dr. N. Sonenberg, Biochemistry Dept., McGill University.

Two cDNA probes for enzymes of folate metabolism, available in our laboratory, were used as controls: a 2 kb cDNA for human MTHFD (methylenetetrahydrofolate dehydrogenase

(Hum et al 1988)), and a 1.3 kb cDNA for human MTHFR (methylenetetrahydrofolate reductase (Zhou et al 1990)).

For radiolabelling, inserts were isolated from an agarose gel by Geneclean (Bio101) and labelled to a specific activity of  $10^9$  cpm/ $\mu$ g using random primed oligonucleotides (Pharmacia).

### **Northern Analysis**

Total cellular RNA was isolated by the guanidinium isothiocyanate-CsCl procedure of Chirgwin et al (1979). Poly A<sup>+</sup> RNA was isolated as in Sambrook et al (1989). The poly A<sup>+</sup> RNA was electrophoresed on a formaldehyde agarose gel, and blotted in 10XSSC to nitrocellulose membranes. The membranes were hybridized overnight with  $10^7$  cpm of probe in a solution of 55% (v/v) formamide, 5XSSC, 1XDenhardts, 20 mM sodium phosphate (pH 7.0), 10% dextran sulfate, and 250  $\mu$ g/ml salmon sperm DNA. The filters were washed 4 times for 5 mins each at room temp in 2XSSC, 0.1% SDS, and once for 15 mins at 50°C in 0.1XSSC, 0.1% SDS.

### **Nuclear Run-on Experiments**

Nuclei were prepared by a modification of Marzluff et al (1985). Cell pellets were collected and resuspended at a concentration of  $5-50 \times 10^6$  cells/ml in Buffer 1 (0.32M sucrose, 5mM MgCl<sub>2</sub>, 10mM Tris-HCl, pH 8.0, 1mM EGTA, 1mM dithiothreitol (DTT), 0.1mM PMSF, 0.1% Triton X-100). Cells

were homogenized and an equal volume of Buffer 2 (Buffer 1 + 2.0 M sucrose) was added. The mixture was layered over a cushion of Buffer 2 and centrifuged at 30,000g for 45 mins at 4° C in a SW50.1 rotor. The nuclear pellet was resuspended in Buffer 3 (25% glycerol, 50mM Tris-HCl, pH 8.0, 0.1mM EGTA, 5.0 mM DTT, 0.1mM PMSF). Aliquots of 100 $\mu$ l were frozen immediately in liquid nitrogen and stored at -70° C. Nuclear run-on assays were carried out according to Ausubel et al (1989) with modification. 200 $\mu$ l of frozen nuclei were thawed and added to 160 $\mu$ l of 2X reaction buffer (10mM Tris-HCl, pH 8.0, 5mM MgCl<sub>2</sub>, 0.3M KCl) plus 10 $\mu$ l of <sup>32</sup>-P labelled ribonucleotide triphosphate GTP,UTP (800 Ci/mmol, 10mCi/ml, NEN) and 10 $\mu$ l of 100mM ATP and CTP (Pharmacia). Reactions were incubated at 30°C, 30 mins, with shaking. RNA was then purified according to Ausubel et al (1989). The human OAT cDNA was run on an agarose gel, and transferred to nitrocellulose or linearized with EcoRI and dotted to nitrocellulose according to Sambrook et al. (1989). Pre-hybridization was carried out for two hours at 42°C in buffers used for Northern analysis. Radiolabelled RNA was added for 48 hours, followed by washing twice in 2XSSC for 1 hr at 65° C. The filters were then incubated at 37°C for 30 mins in 2XSSC and 50  $\mu$ g/ml RNaseA, and washed for 30 mins at 65°C in 0.2% SSC before exposure to X-Ray film.

### **Polysome Gradients**

Approximately  $5-50 \times 10^6$  cells were lysed and processed according to Mullner et al. (1989). Separation of polysomes from the post-mitochondrial supernatant was carried out on a 10 ml linear 10%-40% sucrose gradient, with a 2 ml sample volume. After centrifugation, gradients were separated into 12x1ml fractions, and treated with 30mM EDTA for 10 mins on ice. RNA was isolated from each fraction by diluting with an equal volume of  $H_2O$ , followed by phenol/chloroform/isoamyl alcohol extraction. RNA was precipitated overnight in ethanol at  $-20^\circ C$  and resuspended in  $100\mu l H_2O$ .

For experiments in which EDTA was used to chelate the Mg ions and release RNA from polysomes, the cell extracts were treated with 30mM EDTA for 10 mins on ice prior to loading on the gradient (Young et al 1979). For the experiments in which cycloheximide was used to slow the rate of elongation, cells were treated in culture with  $0.75 \mu g/ml$  cycloheximide for 30 mins., followed by rapid cooling on ice and harvesting of cells (Yenofsky et al 1982). These conditions had been pre-determined to reduce total protein synthesis by approximately 60% in our system (see Methods below for estimation of total protein synthesis).

#### **Reverse Transcription and Polymerase Chain Reaction (PCR)**

In a volume of  $20\mu l$ , one half of the collected RNA from each polysome fraction was incubated with 1 nmol random hexanucleotide primers (Pharmacia), 0.5U RNasin (Promega),  $4\mu l$

5X Buffer (250mM Tris-HCl, pH 8.3, 40mM MgCl<sub>2</sub>, 150mM KCl, 50mM DTT), 0.5mM each dNTP, and 1.0μl reverse transcriptase (100U MMLV enzyme, BRL) at 37°C for 1 hr. Amplification was performed in 100μl containing the 20μl reverse transcriptase reaction and 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM MgCl<sub>2</sub>, 0.01% gelatin (Sigma), 0.2mM each dNTP, 0.2 μM each PCR primer and 2.5 U Taq Polymerase (BRL). Samples were heated at 94° C for 7 mins, 55° C for 2 min, and 72°C for 2 min, followed by 40 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min. For a description of the primers and probes, see fig.7 and table 1.

#### **Analysis of PCR Products**

For hybridization with oligonucleotides, 50μl PCR products were added to 100μl of denaturing solution (0.4N NaOH, 25mM EDTA), heated to 65°C for 10 mins, and applied to a Genetran-45 membrane (Plasco Inc. Woburn, MA.) on a dot-blot manifold (BioRad). Each well was washed with 100μl 20x SSPE (3M NaCl, 20mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, and 20mM EDTA) and the DNA fixed to the membrane by exposure to 254nm UV light for 30 seconds, followed by baking at 80°C for 20 minutes.

The membranes were prehybridized at 37°C for 15 minutes in 10 mls of 5x SSPE, 0.5% SDS, and 5x Denhardts. Hybridization was performed for 1 hour at 2°C below the T<sub>m</sub> in 5xSSPE, 0.5%SDS, 5x Denhardts, plus 50ng of 5' end-labelled probe, prepared according to Sambrook et al (1989). The

membranes were washed for 10 minutes at the  $T_m$  in 2xSSPE, 0.1%SDS. The results were visualized by autoradiography. Membranes were stripped by washing in 0.5M NaOH, 1M NaCl, 100°C, for 30 minutes, followed by a 10 minute wash in 1M Tris, pH 7.4.

#### **Determination of Total Protein Synthesis**

Cells were washed 2x in PBS, placed in Eagle's Minimal Essential Medium (-methionine, Flow Laboratories) for 1 hour. Labelling was carried out for 3 hours with 800 $\mu$ Ci of Trans  $^{35}$ S-label(L-methionine/L-cysteine;ICN) in DMEM, 15% fetal calf serum. Cycloheximide was added at the indicated concentration and for the indicated length of time. After the cycloheximide treatment, cells were placed on ice, pelleted, and lysed in 100mM methionine, TBS, 1% Triton and 100mM PMSF. 10 $\mu$ l (1/50<sup>th</sup>) of the lysate was precipitated with TCA according to Sambrook et al (1989) and the radioactive proteins were quantitated by liquid scintillation counting.

#### **Electroporation**

RB355 cells ( $1 \times 10^7$ ) were electroporated using the IBI Gene Zapper. The cells were mixed with 30 $\mu$ g of XhoI-linearized pMV7-4E or pMV7-4E(A) expression vectors; these vectors contain the full-length coding sequence for a wild-type and mutant form, respectively, of the murine eIF-4E (Lazaris-Karatzas et al 1990). The cells were pulsed 2X at a

**Figure 7.** Amplification primers and oligonucleotide probes for analysis of OAT mRNA by PCR.

Amplification primers P1 (in exon 1) and P3 (in exon 3) yield PCR products of 308 bp and 221 bp for the unspliced and spliced OAT mRNA, respectively. PCR products are identified by hybridization with P2 (an exon 2-specific oligonucleotide) to detect the unspliced OAT mRNA, or P4 (an oligonucleotide containing the last 10 bases of exon 1 and the first 10 bases of exon 3) to detect the spliced OAT mRNA. The picture at the right shows ethidium bromide stained PCR products resulting from amplification of reverse transcribed RB355 RNA (lane 2) or Y79 RNA (lane 3) run on a 3% agarose gel. Lane 1 is  $\phi$ X 174RF DNA/HaeIII molecular weight markers. 1-2-3 and 1-3 refer to the unspliced and spliced OAT mRNA respectively. MTHFD is an 100 bp PCR fragment resulting from amplification of reverse transcribed RNA using MTHFD (20) specific primers. Southern analysis confirmed the specific hybridization of P2 and P4 to the larger and smaller PCR products, respectively (data not shown).

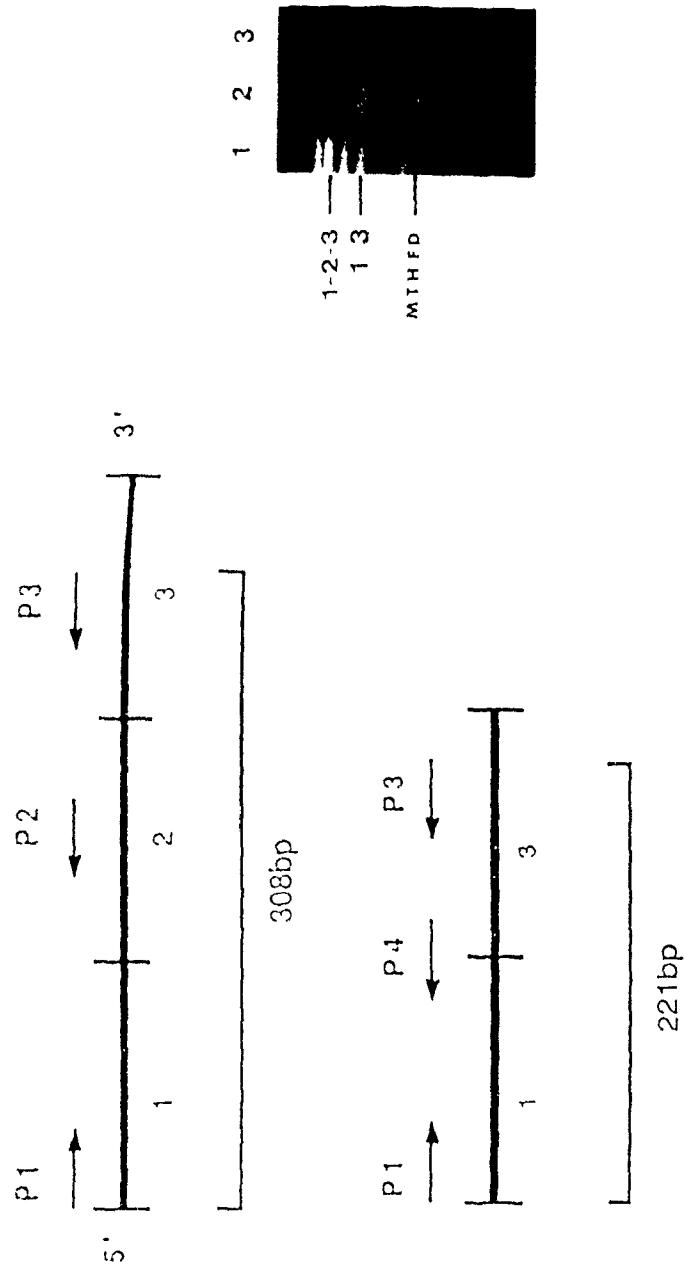


Table 1. OAT Specific Oligonucleotide Primers and Probes

| Name | bp <sup>*</sup>                     | Sequence                      | T <sub>m</sub> (°C) <sup>#</sup> |
|------|-------------------------------------|-------------------------------|----------------------------------|
| P1   | (-)162 to (-)142                    | 5'GCTTCATAGACGC<br>CGCGTGTA3' | 68                               |
| P2   | (-)50 to (-)30                      | 5'TGACTATTTTTC<br>TTCCCACG3'  | 52                               |
| P3   | 126-146                             | 5'TTATATTCCTAT<br>CAAAAATG3'  | 52                               |
| P4   | (-)29 to (-)20/<br>(-)126 to (-)117 | 5'AACCACAGAT/CT<br>GACAGCGC3' | 62                               |

\* numbering of bases: (+)1 refers to the ATG methionine initiation codon (Mitchell et al 1988)

# calculated using the formula  $4(G+C) + 2(A+T)$

setting of 300  $\mu$ F capacitance, 400 V which leads to a timeconstant of approximately 30-35 msec. The cells were diluted 10-fold with medium and cultured for 48 hrs. without selection. At 48 hrs., G418 (neomycin) was added at a concentration of 500  $\mu$ g/ml, and cells were selected for at least 10 days prior to harvesting.

### **Primer Extension**

Primer extension was carried out according to Marzluff et al. (1985) using a 21-mer primer complementary to residues 126-146 where bp (+)1 refers to the translation initiation codon (Mitchell et al 1988) (oligo 1). To test for the presence of exon 2, an oligonucleotide (21-mer) complementary to residues (-)98 to (-)78 (Mitchell et al 1988) was used (oligo 2). The primers were 5' end-labelled using [ $\gamma$ - $^{32}$ P] ATP and  $T_4$  polynucleotide kinase (Sambrook et al 1989) to a specific activity of  $10^{10}$  cpm/ $\mu$ g. 0.25 fmol of the labelled primer was hybridized to poly A<sup>+</sup> RNA for 6 hours in 1M NaCl, 25mM Pipes (pH 7.0) at 40°C. Extension was performed for 1.5 hours at 42°C in 50mM Tris (pH 8.3), 75mM NaCl, 10mM dithiothreitol, 3mM MgCl<sub>2</sub>, and 0.5mM dNTPs in the presence of reverse transcriptase (BRL). The products were electrophoresed on a 5% (oligo 1), or a 20% (oligo 2) polyacrylamide gel and visualized by autoradiography.

### **Statistical Analysis**

The data was analyzed by the student t test or two-way analysis of variance test. Mean  $\pm$  standard deviation (s.d.) is presented where appropriate.

#### IV. RESULTS

##### A. Differences in Translational Efficiency of OAT Between Retinoblastomas RB355 and Y79

###### A.1. Basal levels of OAT activity, protein, and mRNA

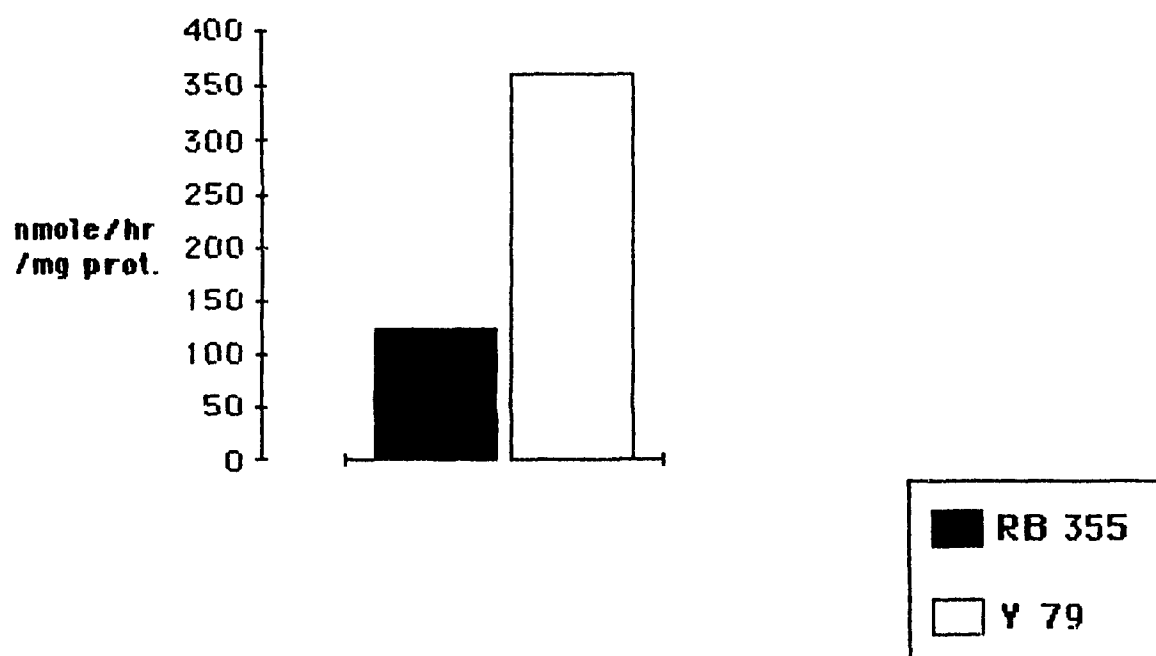
OAT enzyme activities were measured in duplicate on 3 occasions, using a modified assay of Ohura et al (1983); mean OAT activities ( $\pm$  standard deviation) were  $365 \pm 6$  and  $126 \pm 4$  nmol/hr/mg protein (fig.8,top graph) for Y79 and RB355, respectively ( $p < .001$ ). These values are in the range reported for OAT enzyme activities in rat, pig (Ratzlaff et al 1983), and cat (Valle et al 1981) liver and whole retina.

Immunoblotting with OAT-specific antiserum (fig.9,top) and quantitation by densitometry (fig.8,bottom) was used to assess steady - state levels of OAT protein in RB355 and Y79. The Y79 strain contained 3-fold more OAT protein than the RB355 strain in three separate experiments performed in triplicate ( $15.1 \pm 0.4$  vs.  $5.1 \pm 0.2$ ,  $p < .001$ ). The same immunoblots, reacted with actin antiserum as a control (fig 9,bottom), revealed that actin protein was present at similar levels in both strains ( $11.7 \pm 0.3$  vs.  $11.2 \pm 0.2$ ,  $p > .05$ ). This suggests that the differences observed are OAT-specific, rather than a universal decrease in protein synthesis in the RB355 strain. The 3-fold difference in immunoreactive OAT

**Figure 8.** OAT enzyme activity and immunoreactive protein levels in retinoblastomas.

OAT basal activity (top) and immunoreactive protein (bottom) were measured as described in Methods. The top graph depicts the mean of 6 determinations (3 experiments, each performed in duplicate (40 $\mu$ g and 80 $\mu$ g protein)). The bottom graph indicates the values for 3 separate determinations (20 $\mu$ g, 40 $\mu$ g, and 80 $\mu$ g protein) for each strain.

### OAT Basal Activity



### Immunoreactive Protein Basal Levels

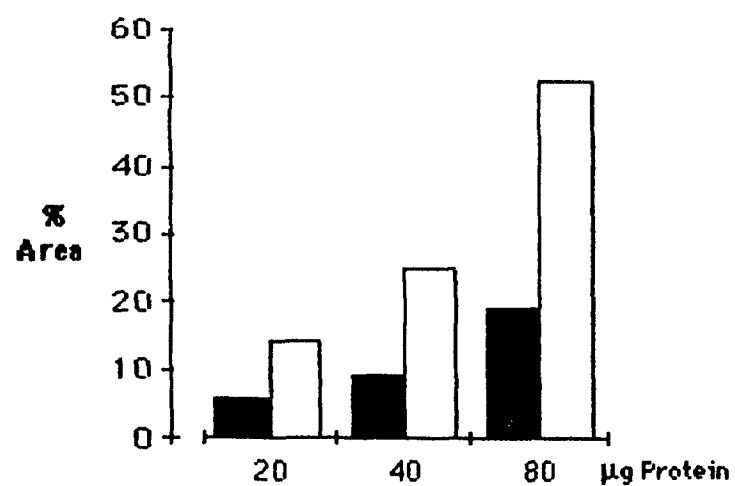
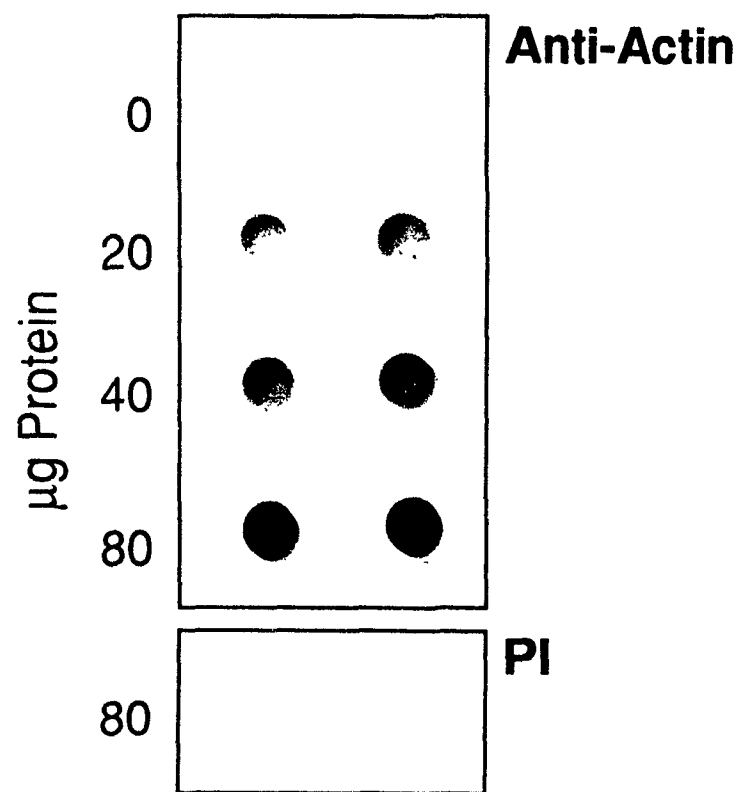
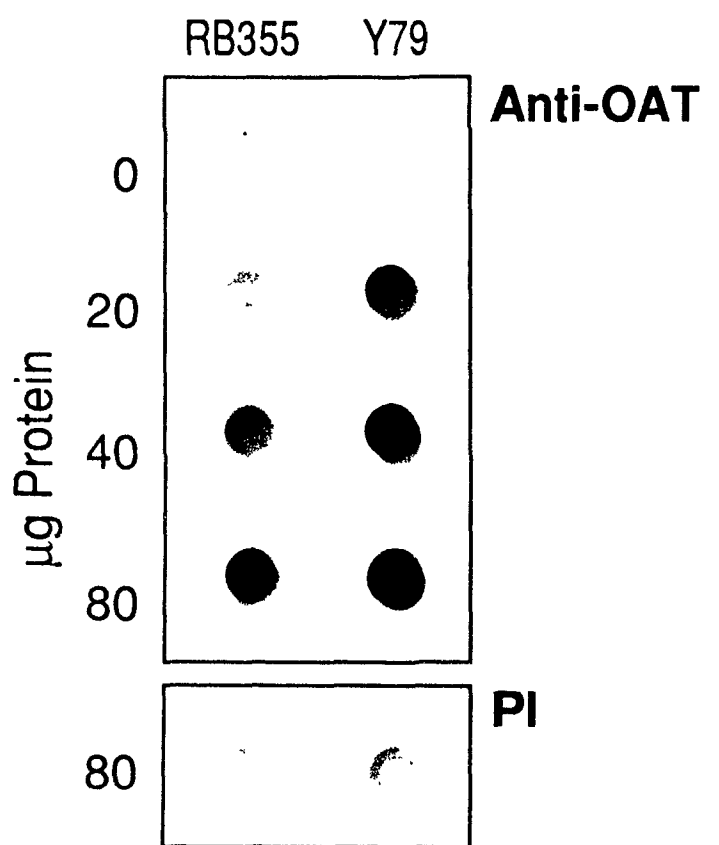


**Figure 9.** OAT and actin immunoreactive protein levels in retinoblastomas.

20, 40 and 80 $\mu$ g total protein were dotted to duplicate membranes. In the top half of the figure, the membrane was incubated with OAT antiserum (anti-OAT). In the bottom half, actin antiserum was used (anti-actin). PI represents 80 $\mu$ g cell protein incubated with pre-immune sera, as a control for non-specific reactivity.



protein corresponded well to the difference seen for OAT enzyme activity.

Steady - state levels of OAT mRNA in retinoblastomas RB355 and Y79 were measured by Northern blotting, using a human OAT cDNA. The analysis revealed a 2.3 kb mRNA (fig. 10,top) which, after correction for amounts of mRNA loaded, upon densitometric analysis, was shown to be present in approximately equal amounts in both strains; the size of the mRNA corresponded to that previously reported (Inana et al 1986, Mitchell et al 1988). As a control for RNA amounts loaded per lane, each blot was reprobbed with a cDNA for the trifunctional enzyme of folic acid metabolism, MTHFD (fig.10,bottom) (Hum et al 1988).

#### A.2. OAT transcription in retinoblastomas

The data from Northern blotting suggested that steady state levels of OAT mRNA were similar in the two strains. To ensure that the difference in the level of OAT expression between strains was not due to a difference in OAT transcription, a nuclear run-on analysis was performed. Fig. 11 shows an autoradiogram of two nitrocellulose filters with varying amounts of immobilized OAT cDNA and the Bluescript vector. Each filter was probed with radiolabelled RNA transcribed by isolated nuclei from either Y79 (lanes 1 and 2) or RB355 (lanes 3 and 4). To control for the amount of cDNA

**Figure 10.** Northern analysis of RB355 and Y79 poly A<sup>+</sup> RNA.

10 $\mu$ g of poly A<sup>+</sup> RNA was electrophoresed on a 1.2% formaldehyde-agarose gel, transferred to a nitrocellulose membrane, and hybridized with a radiolabelled cDNA as described in Materials and Methods. The upper panel depicts the autoradiogram after hybridization with an OAT cDNA. The lower panel depicts the same Northern blot after stripping and reprobing with a MTHFD cDNA as a control for amount of RNA loaded per lane. The autoradiograms were scanned with a LKB densitometer and a ratio of OAT mRNA : MTHFD mRNA was calculated in order to compensate for differences in amount of RNA loaded; an examination of the ratios of OAT mRNA: MTHFD mRNA indicated similar values between strains (2.4 vs 2.3).

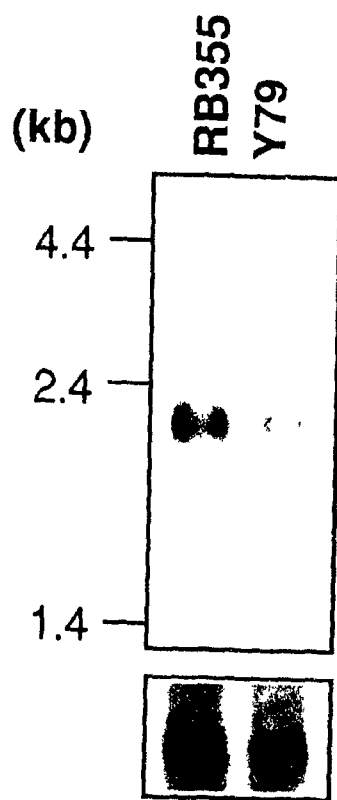
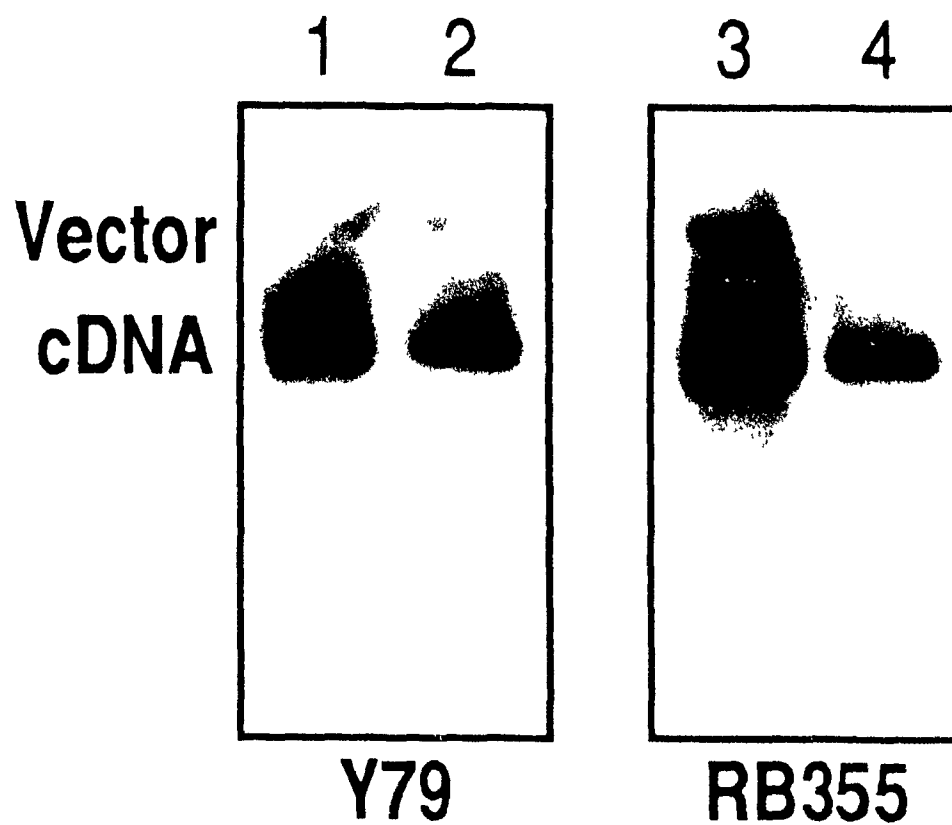


**Figure 11.** Nuclear run-on analysis of the OAT gene.

Plasmid DNA containing the OAT cDNA (500 ng. in lanes 1 and 3; 50 ng. in lanes 2 and 4) was digested with EcoRI and run on a 1% agarose gel. The gel was transferred in 20X SSC to nitrocellulose membranes and baked for 1 hr at 80°C. <sup>32</sup>P-labelled RNA from Y79 ( $1.8 \times 10^7$  cpm; left panel) and from RB 355 ( $1.7 \times 10^7$  cpm; right panel) nuclei was hybridized to the membranes as outlined in Methods. The autoradiograms were scanned by densitometry; the ratios of lanes 1:3 and lanes 2:4 were calculated.



immobilized on each filter, the filters were stripped and reprobed with the alternative radiolabelled RNA from a second nuclear run-on. The autoradiograms (before and after stripping of the filters) were scanned with a densitometer and the ratio of lanes 1:3 as well as lanes 2:4 were determined. Since the ratios were similar, it is likely that the two strains transcribe the OAT gene at equivalent rates. However, since immunoreactive OAT and enzyme activity were 3-fold higher in Y79, there must be a post-transcriptional mechanism for the regulation of OAT in retinoblastomas.

#### A.3. Polysome analysis of OAT mRNA in retinoblastomas

To determine the translational efficiency of the OAT mRNA in both strains, the distribution of OAT mRNA in polysomes was examined. Polysomes were isolated and fractionated on a sucrose gradient. Fig.12 is a graph depicting the  $A_{260}$  profile of the 12 polysome fractions, indicating that fractions 1-2 contain the ribosomal subunits and fractions 3-12 contain polysomes. Actively-translated mRNAs (with many bound ribosomes) should be heavier and fractionate towards the bottom of the sucrose gradient. Fig. 13 shows the sedimentation profile of the spliced OAT mRNA species (without exon 2) in RB355 and Y79 polysomes. The spliced OAT mRNA is present in the lighter polysome fractions in RB355, as compared to that of Y79 (fractions 1-6 vs. fractions 5-9). To

**Figure 12.** Optical density ( $A_{260\text{nm}}$ ) profile of Y79 polysome gradient.

Approximately  $5 \times 10^6$  cells were lysed, processed, and fractionated on a 10-40% linear sucrose gradient. The optical density of the resulting 12x1ml fractions was then measured at 260nm. Fraction 1 represents the top of the gradient, fraction 12 the bottom.

O.D. (260nm) vs. Fraction

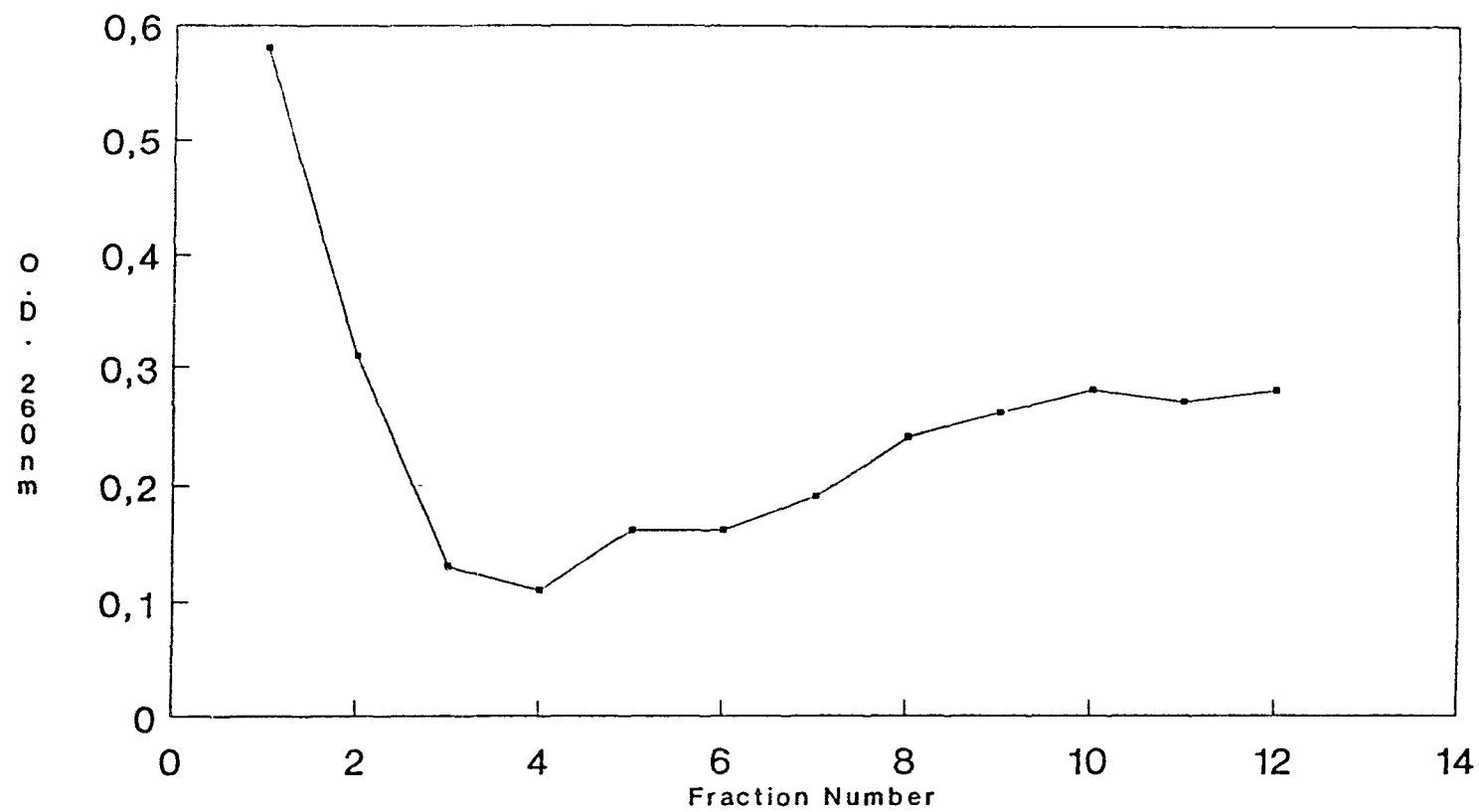
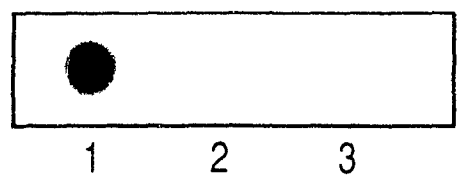
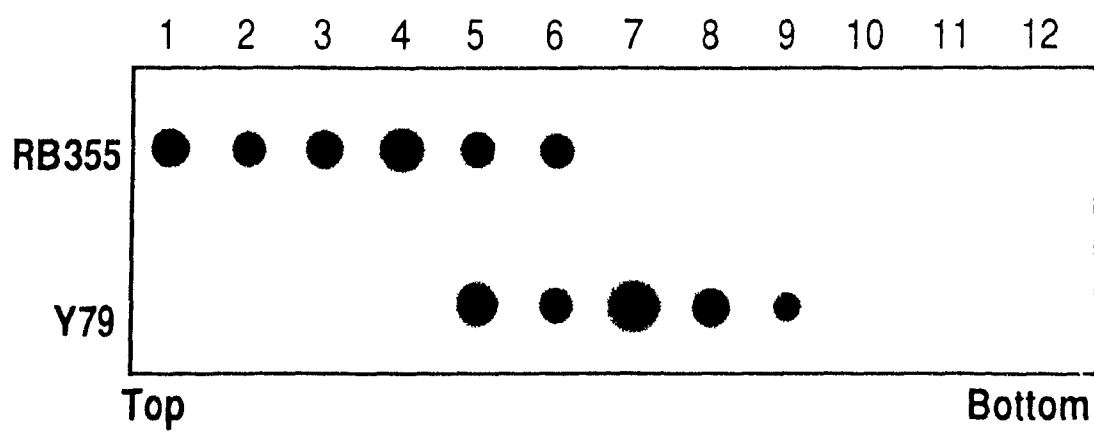


**Figure 13. Polysome profile of spliced OAT mRNA**

Polysomes were isolated and fractionated on a 10 ml linear 10% - 40% sucrose gradient. The RNA in each fraction was reverse transcribed and amplified by PCR. PCR products were analyzed by dot blots. The nitrocellulose filter was hybridized with P4 (see Methods), to detect the spliced OAT mRNA. The bottom panels of each figure depict controls for the PCR and for hybridization: dot 1 is 20  $\mu$ l of a PCR reaction resulting from reverse transcription of 5  $\mu$ g total RNA from Y79, using OAT primers and probes; dot 2 represents the same analysis with MTHFR primers and probes; dot 3 represents the same analysis without RNA to rule out PCR contamination.



ensure the integrity of the gradients in each strain, the procedure was repeated using primers and oligonucleotide probes for an enzyme of folate metabolism, designated MTHFR. Fig. 14 shows the sedimentation of MTHFR mRNAs in polysomes of RB355 and Y79; no major differences in the sedimentation profile were observed between the strains. These data suggest that RB355 translates OAT less efficiently than Y79. Furthermore, inefficient translation is not a general property of the RB355 strain, since MTHFR appears to be actively translated in both strains.

Since EDTA, a chelator of Mg ions, should dissociate mRNA from ribosomes, treatment of the polysomes with 30 mM EDTA prior to fractionation should release the RNA to the lighter fractions. Fig. 15 indicates that this phenomenon occurred in both strains, supporting the notion that the RNA had been sequestered on polysomes rather than in ribonucleoprotein particles (Ajtkhozin et al 1973).

#### A.4. Efficiency of translational initiation of OAT mRNA in RB355 and Y79

To determine if the difference in translation was due to a difference in initiation vs. elongation, fractionation of polysomes was performed after treatment of intact cells with cycloheximide. The concentration that achieved a 60% reduction in the rate of protein synthesis and the duration of

**Figure 14. Polysome profile of MTHFR mRNA**

Half of the RNA isolated from the polysome preparation in figure 12 was reverse transcribed and amplified by PCR using primers specific for MTHFR. PCR products were analyzed by dot blots. The nitrocellulose filter was hybridized with an oligonucleotide specific for MTHFR (see Methods). The bottom panels of each figure depict controls for the PCR and hybridization: dot 1 is 20  $\mu$ l of a PCR reaction resulting from reverse transcription of 5 $\mu$ g total RNA from Y79, using OAT primers and probes; dot 2 represents the same analysis with MTHFR primers and probes; dot 3 represents the same analysis without RNA to rule out PCR contamination.

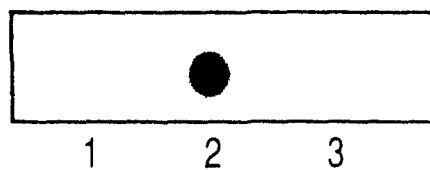
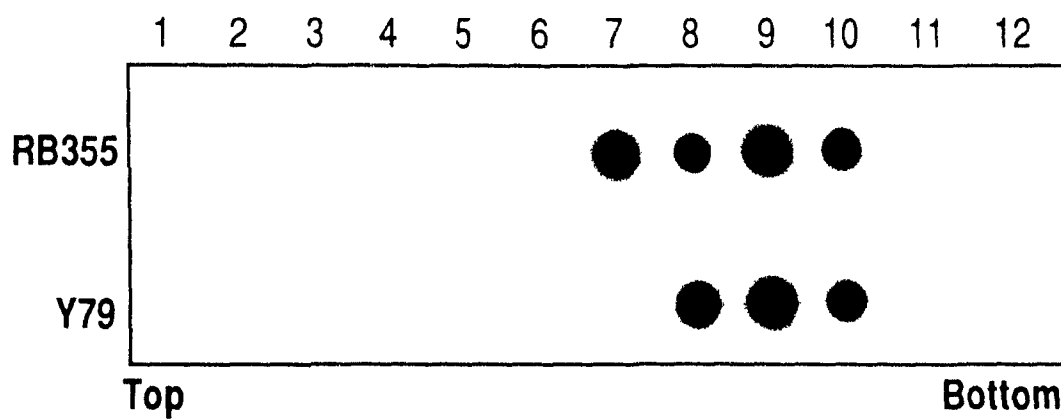
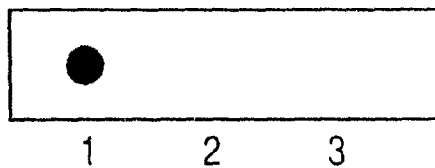
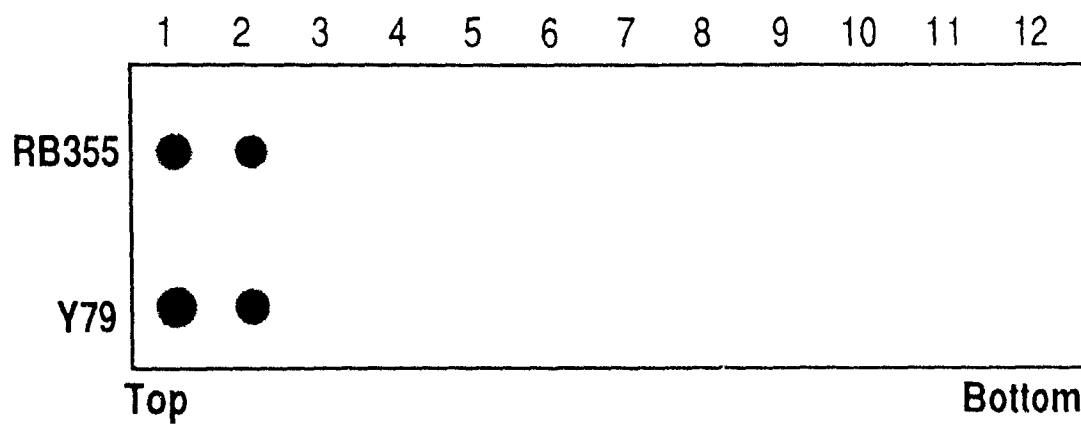


**Figure 15.** Polysome profile of spliced OAT mRNA after EDTA treatment.

Polysomes were isolated and fractionated on a 10 ml linear 10% - 40% sucrose gradient. The RNA in each fraction was reverse transcribed and amplified by PCR. PCR products were analyzed by dot blots. See Methods for details. A polysome preparation was divided into 2 equal portions: one portion was treated with 30mM EDTA for 10 mins. at 4°C before fractionation while the second portion was not pre-treated. The filter was probed with P4 to identify the spliced OAT mRNA; only the EDTA-treated polysome profile is shown here since the untreated profile was essentially as depicted in Figure 13. The bottom panels of each figure depict controls for the PCR and hybridization: dot 1 is 20  $\mu$ l of a PCR reaction resulting from reverse transcription of 5 $\mu$ g total RNA from Y79, using OAT primers and probes; dot 2 represents the same analysis with MTHFR primers and probes; dot 3 represents the same analysis without RNA to rule out PCR contamination.



treatment was determined experimentally (table 2). At this level of inhibition, this antibiotic reduces the rate of elongation to the extent that elongation becomes the rate-limiting step of translation (Fan et al 1970, Lodish et al 1971). Thus, an RNA that initiates translation and elongates normally, will load more ribosomes, in the presence of cycloheximide, and will appear in a denser fraction of the polysome gradient. However, a shift in the gradient will not be observed if the RNA is blocked during elongation. Fig. 16 shows the results of cycloheximide treatment on the polysome profile of the spliced OAT mRNA. In both strains, cycloheximide treatment caused a shift in the gradient. In RB355, OAT mRNA was found in two additional fractions, 6 and 7; in Y79, OAT mRNA shifted into 3 additional fractions, fractions 10-12. These results suggest that the OAT spliced mRNA elongates normally in both strains. The fact that it undergoes a greater shift in the Y79 strain is compatible with more efficient initiation in Y79. The same experiment performed on MTHFR mRNA, as a control, revealed that this mRNA shifted to the bottom of the gradient, fractions 11-12, in both strains (fig. 17).

**Table 2.** Inhibition of protein synthesis by cycloheximide.

Cells were incubated in culture with  $^{35}\text{S}$ -methionine and cycloheximide for the given times and concentrations shown in table 2. The labelling, lysis, precipitation of proteins, and counting was performed as in Methods. The % of total protein synthesis was calculated using the zero value as 100%.

Table 2

| cycloheximide<br>concentration | cell<br>strain | time of<br>exposure(mins.) | % of total<br>protein syn. |
|--------------------------------|----------------|----------------------------|----------------------------|
| 0.75 $\mu\text{g/ml}$          | Y79            | 0                          | 100(42,987cpm)             |
|                                |                | 15                         | 63                         |
|                                |                | 30                         | 36                         |
|                                |                | 60                         | 38                         |
|                                | RB355          | 0                          | 100(36,237cpm)             |
|                                |                | 15                         | 47                         |
|                                |                | 30                         | 51                         |
|                                |                | 60                         | 41                         |
| 1.25 $\mu\text{g/ml}$          | Y79            | 0                          | 100(36,323cpm)             |
|                                |                | 15                         | 62                         |
|                                |                | 30                         | 18                         |
|                                |                | 60                         | 29                         |
|                                | RB355          | 0                          | 100(40,044cpm)             |
|                                |                | 15                         | 20                         |
|                                |                | 30                         | 21                         |
|                                |                | 60                         | 23                         |

**Figure 16.** Polysome profile of spliced OAT mRNA before and after cycloheximide treatment.

(-) Polysome profile of untreated RB355 and Y79 cells  
(+) Polysome profile of RB355 and Y79 after treatment in culture with 0.75  $\mu$ g/ml cycloheximide for 30 min. Fractionation, PCR, and dot blot analysis were performed as in Fig. 13. The filter was probed with P4 to identify the spliced OAT mRNA.

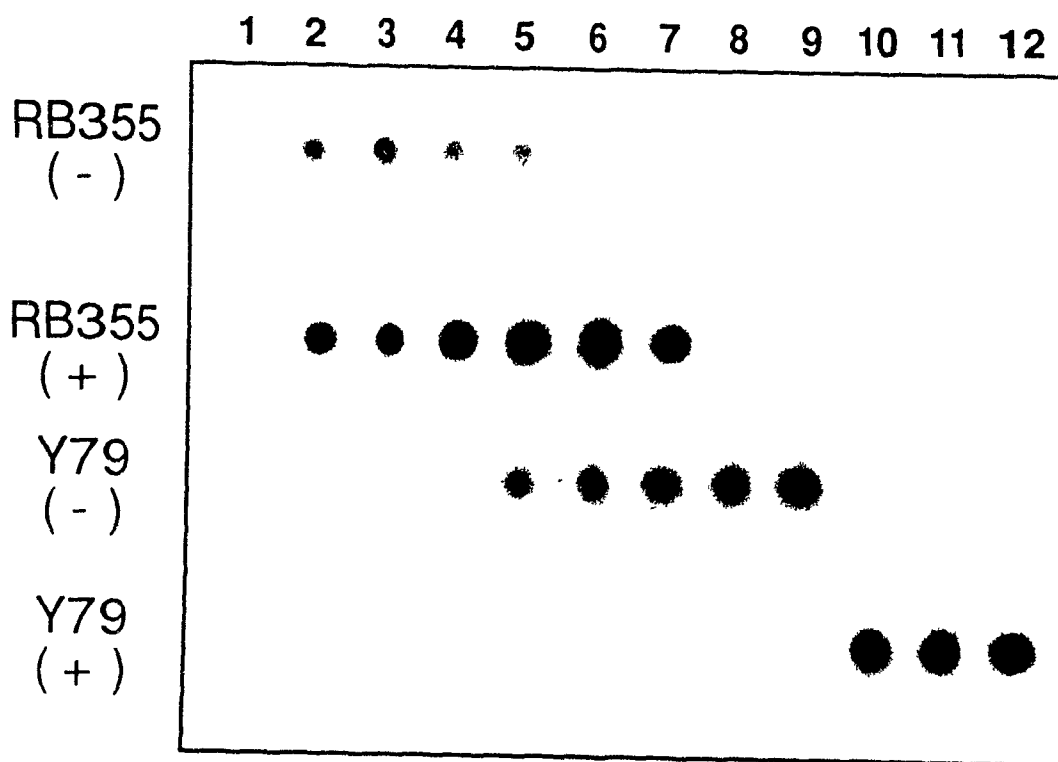
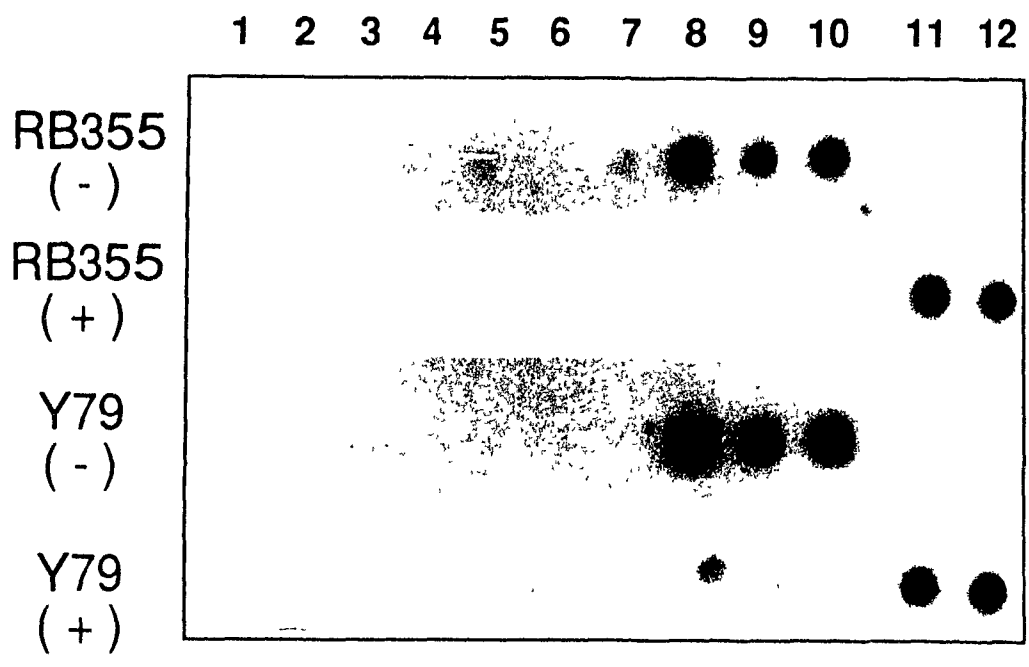


**Figure 17.** Polysome profile of MTHFR mRNA before and after cycloheximide treatment.

(-) Polysome profile of untreated RB355 and Y79 cells.  
(+) Polysome profile of RB355 and Y79 after treatment in culture with 0.75  $\mu$ g/ml cycloheximide for 30 mins. Fractionation, PCR, and dot blot analysis were performed as in Fig. 14. The filter was probed with an MTHFR oligonucleotide to identify the MTHFR mRNA.



## B. Translational Control of OAT by eIF-4E

### B.1. eIF-4E mRNA and protein levels in RB355 and Y79

To determine the mechanism of the translational difference between strains, we explored the possibility that the RB355 strain might have a reduced amount of a translation initiation factor. We therefore examined the levels of eIF-4E, the cap binding protein, which is the rate-limiting factor of eucaryotic translational initiation (Hiremath et al 1985, Duncan et al 1987, Hiremath et al 1989). Fig. 18 depicts the levels of eIF-4E mRNA (top left panel) and of eIF-4E immunoreactive protein (top right panel) in both strains. The RB355 strain has reduced levels of eIF-4E, based on the Northern and the Western analyses.

### B.2. Transfection of RB355 with eIF-4E and eIF-4E(A)

If the decreased OAT translation in RB355 was a consequence of limited amounts of eIF-4E, overexpression of this initiation factor in RB355 should enhance OAT translation. Electroporation of RB355 was performed, using expression vectors pMV7-4E or pMV7-4E(A) (fig.19). Both constructs contain the retroviral expression vector pMV7 (Kirschmeier et al 1988) with either the full-length murine eIF-4E coding sequence (pMV7-4E (Lazaris-Karatzas et al

**Figure 18.** Analysis of eIF-4E mRNA and protein levels in retinoblastomas.

The top left panel depicts a Northern blot of 25  $\mu$ g poly A<sup>+</sup> RNA from Y79 and RB355, electrophoresed on a 1% formaldehyde-agarose gel, transferred to a nitrocellulose membrane, and hybridized with a radiolabelled full-length (1.6 kb) eIF-4E cDNA insert from pMV7-4E. The blot was stripped and reprobed with an actin cDNA (internal control) which indicated that equal amounts of RNA were loaded per lane (bottom left panel). The top right panel depicts a Western blot of 100  $\mu$ g protein per lane, electrophoresed on a discontinuous 15% polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with antiserum against eIF-4E. Antiserum against actin was used as an internal control on a separate blot to ensure that the amount of protein in each lane was similar (bottom right panel).

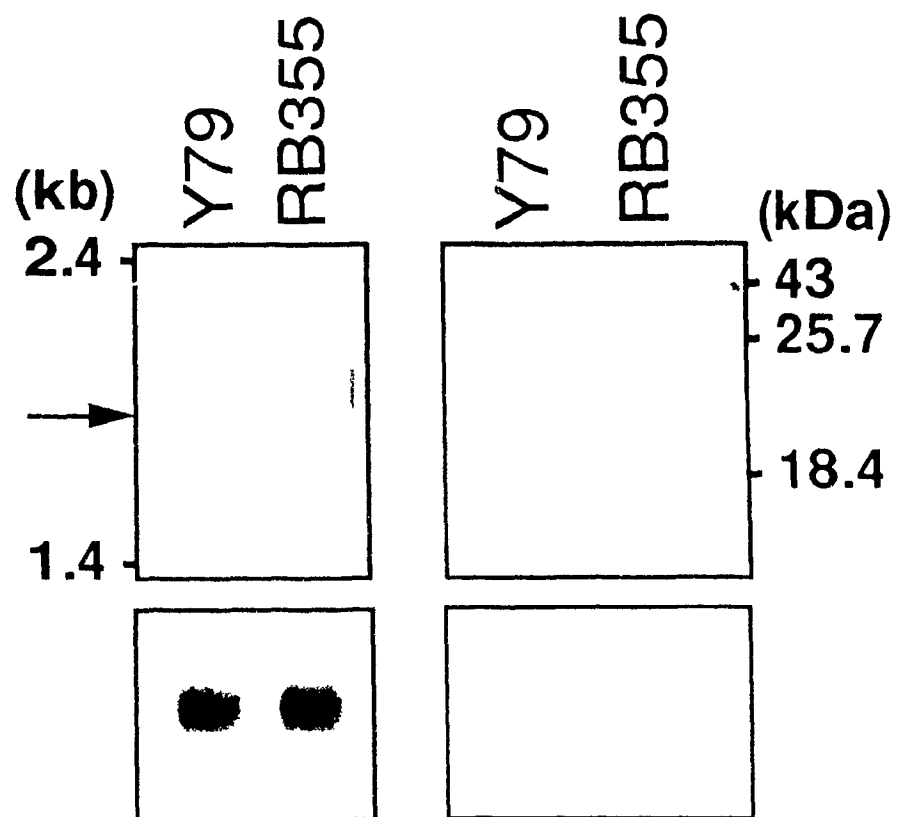
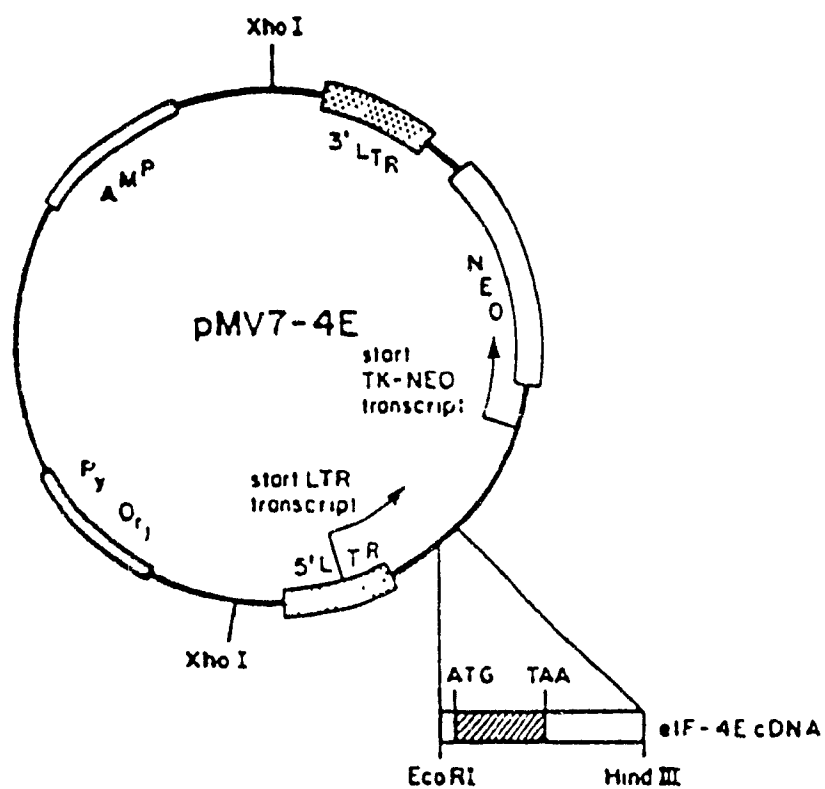


**Figure 19. pMV7-4E expression construct.**

pMV7 contains the 5' and 3' LTR of the Muloney Murine Leukemia Virus, and the bacterial neomycin resistance gene under the control of the thymidine kinase promoter (Kirschmeier et al 1988). pMV7-4E contains the full-length murine eIF-4E coding sequence. pMV7-4E(A) is identical to pMV7-4E, except that serine codon 53 has been mutated to alanine.



1990)), or the full-length murine eIF-4E sequence in which the serine 53 codon has been mutated to alanine (pMV7-4E(A) (Lazaris-Karatzas et al 1990)). The serine 53 codon has been shown to be the main site of phosphorylation (Rychlik et al 1987), and phosphorylation is required to activate the function of eIF-4E (De Benedetti et al 1990). Western analysis using antiserum against eIF-4E (fig.20; lanes 2 and 3 as compared to lane 1) demonstrated similar increases (~3-fold) of eIF-4E protein in cells transfected with the 2 constructs. A similar increase (~3-fold) was observed when this construct was used to transform NIH-3T3 cells (Lazaris-Karatzas et al 1990).

### B.3. Modulation of OAT expression by eIF-4E

Fig.21 depicts the polysome<sup>1</sup> profile for the OAT spliced mRNA in the RB355 cells transfected with wild-type eIF-4E, or mutagenized eIF-4E(A). Overexpression of wild type eIF-4E shifts the OAT mRNA to the heavier portion of the gradient, fractions 7-9, as compared to untransfected RB355 (fractions 1-6; see Figure 13). The fractional distribution of OAT mRNA in the transfected RB355 strain is now quite similar to that in the untransfected Y79 strain (see Figure 13). Overexpression of mutagenized eIF-4E(A) does not result in a shift toward the bottom of the gradient; in fact, the profile is somewhat suggestive of a shift toward lighter fractions.

**Figure 20.** eIF-4E and eIF-4E(A) levels in RB355 before and after transfection.

Cells were lysed, extracts were prepared, and Western analysis was performed according to Methods. 100 $\mu$ g of total cell protein from non-transfected RB355 (lane 1), RB355 transfected with eIF-4E (lane 2), or RB355 transfected with eIF-4E(A) (lane 3) was electrophoresed on a discontinuous 15% polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with antisera against eIF-4E (top panel). Antisera against actin was used as an internal control on a separate blot to ensure that the amount of protein in each lane was similar (bottom panel).

1 2 3

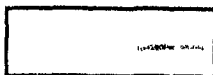
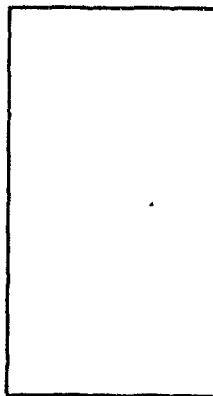
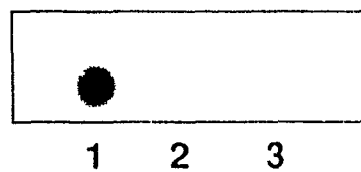
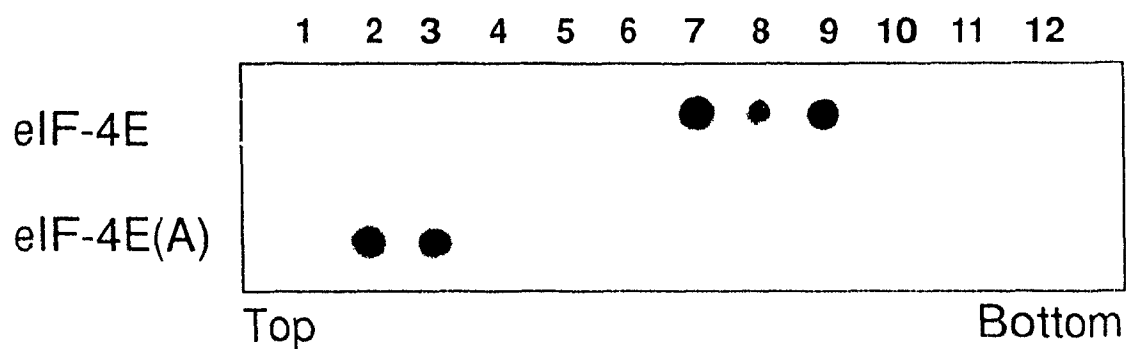


**Figure 21.** Polysome profile of OAT spliced mRNA in RB355 transfectants.

This figure depicts the polysome profile of RB355 cells that had been transfected with the wild-type cDNA (eIF-4E) or the mutagenized cDNA (eIF-4E(A)). Fractionation, PCP, and RNA blot analysis were performed as in Figure 13. The filter was hybridized with P4 to identify the spliced OAT mRNA.



Overexpression of the mutated eIF-4E(A) in NIH-3T3 cells extended the doubling time for the strain, possibly due to a transdominant negative effect on growth (Lazaris-Karatzas et al 1990). A similar phenomenon may have occurred in this experiment.

Polysome analysis of MTHFR mRNA after transfection (fig.22) indicated that there was no change in the position in the polysome gradient (compare to fig.14). This finding suggests that translation of MTHFR mRNA is not limited by the availability of eIF-4E.

Fig. 23, top panel, depicts OAT immunoreactive protein levels in untransfected RB355 and Y79, and in the transfected RB355. The untransfected Y79 strain (lane 2) contains more OAT protein than RB355, as previously shown (fig.9 top). The RB355 strain transfected with eIF-4E (lane 4) contains increased amounts of OAT protein, whereas the RB355 cells transfected with eIF-4E(A) (lane 3) maintained the same levels of OAT protein as the untransfected RB355 (lane 1). The levels of OAT protein in the transfected RB355 strain are now quite similar to the levels in the untransfected Y79 strain; the above-mentioned observation of similar polysome profiles in the two strains after transfection is consistent with equivalent levels of OAT protein. Actin controls (fig.23, bottom panel) indicate that equivalent amounts of total cell protein were loaded per lane.

**Figure 22.** Polysome profile of MTHFR mRNA in RB355 transfectants.

Figure 22 depicts the polysome profile of RB355 cells that had been transfected with the wild-type cDNA (eIF-4E) or the mutagenized cDNA (eIF-4E(A)). Fractionation, PCR, and dot blot analysis were performed as in Figure 14. The filter was hybridized with a MTHFR oligonucleotide to identify MTHFR mRNA.

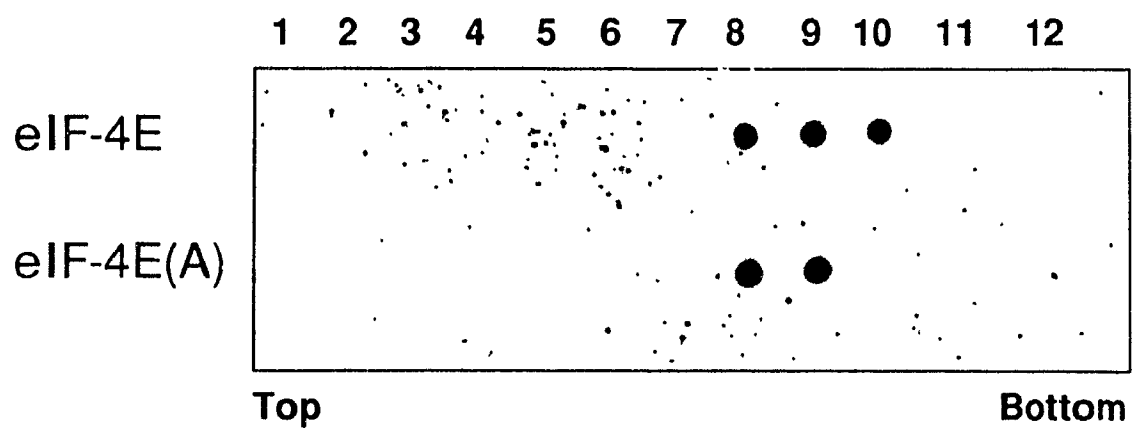
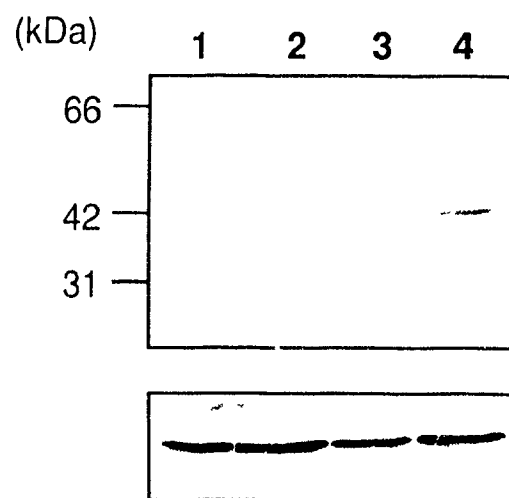


**Figure 23.** OAT immunoreactive protein levels in RB355 transfectants.

Extracts of 80 $\mu$ g protein from the RB355 transfectants were electrophoresed on a discontinuous 10% polyacrylamide gel, transferred to a nitrocellulose membrane and incubated with OAT antiserum (top panel) as per Methods. Lanes 1 and 2 represent untransfected RB355 and Y79 cells, respectively; lanes 3 and 4 represent RB355 transfected with the mutagenized eIF-4E(A) cDNA and the wild-type eIF-4E cDNA, respectively. Antisera against actin was used as an internal control on a separate blot to ensure that the amount of protein in each lane was similar (bottom panel).



### C. Alternative Splicing of OAT mRNA in RB355 and Y79

#### C.1. Primer extension analysis of OAT mRNA

Mitchell et al. (1988) reported the finding of a splice site (exon 2) which was spliced out of all cDNAs studied. To determine whether the OAT mRNA in retinoblastomas used this splice, primer extension analysis was performed using an oligonucleotide specific for exon 3. Two major extension products were observed in both strains (fig. 24). The smaller species,  $\approx 220$  bp, was of the predicted length for the spliced species, considering the published cDNA sequence (Mitchell et al 1988); the second species was 280-310 bp.

The possibility that the larger primer extension species in retinoblastomas was due to the presence of exon 2 in the mature OAT transcript was re-examined by primer extension using an oligonucleotide specific for exon 2 (Mitchell et al 1988). Fig. 25 indicates that in both RB355 and Y79, only one extension product, with an approximate length of less than 100 bp, was synthesized from the exon 2 primer. This finding suggests that only one extension product, the larger species in fig. 24, contains exon 2. Therefore both retinoblastoma strains contain two populations of mature OAT transcripts due to the presence of exon 2 in one of the mRNA species. Because band intensities varied between experiments, no definite conclusions could be rendered regarding the levels of each

**Figure 24.** Primer extension analysis of OAT mRNA in RB355 and Y79.

A synthetic 21-mer oligonucleotide, corresponding to nucleotides (+)126 to (+)146 (exon 3) of the human OAT cDNA (Mitchell et al 1988) was annealed to 1  $\mu$ g (lanes 1 & 3) or 3  $\mu$ g (lanes 2 & 4) of poly A<sup>+</sup> RNA from RB355 (lanes 1 & 2) and Y79 (lanes 3 & 4), and extended with reverse transcriptase, as described in Methods. The products were run on a 5% denaturing polyacrylamide gel and sized using a lambda Hind III marker. Two extension products were observed in each strain corresponding to approximately 220 bp and 300 bp in size.

**RB355 Y79**

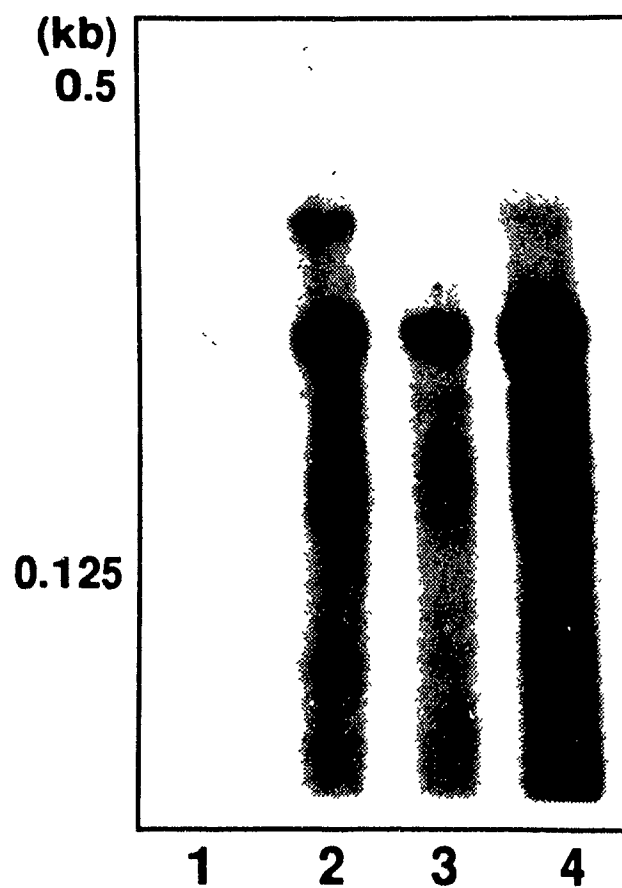
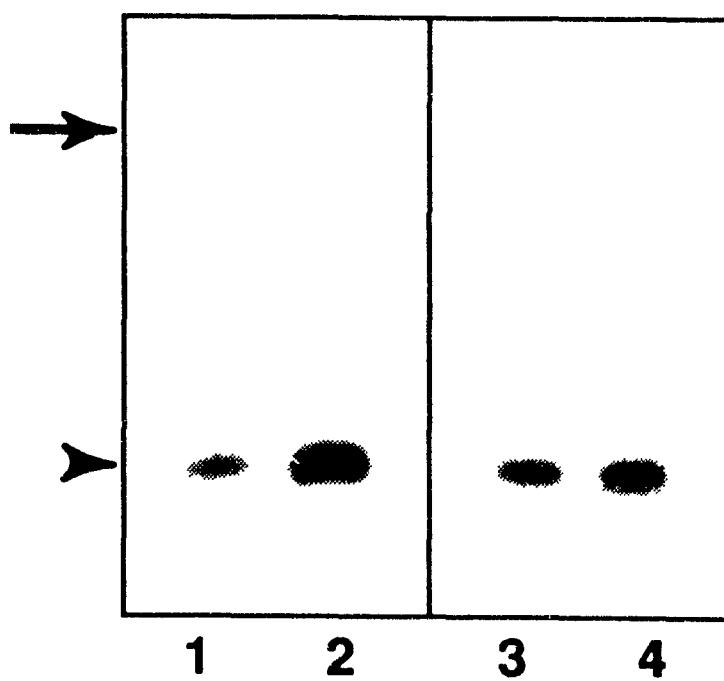


**Figure 25.** Exon 2-specific primer extension analysis in RB355 and Y79.

A synthetic 21-mer oligonucleotide, corresponding to nucleotides (-)98 to (-)78 (exon 2) of the human OAT structural gene (Mitchell et al 1988; (+)1 indicates translation initiation codon) was annealed to 1 $\mu$ g of poly A<sup>+</sup> RNA from RB355 (lane 3) and Y79 (lane 4), and extended with reverse transcriptase. The products were run on a 20% denaturing polyacrylamide gel. A single extension product was seen in each strain at less than 100 bp. Lanes 1 & 2 depict the 5' labelled primers, nucleotides (+)126 to (+)146 (primer used for extension in fig.24) and nucleotides (-)98 to (-)78 (exon 2 primer), respectively. Arrowhead indicates unbound primers and arrow indicates primer-extended products.

RB355 Y79



transcript, although the smaller species was always more abundant in each strain.

#### C.2. Translational efficiency of exon 2-containing OAT mRNAs

To assess the relative rate of translation of the unspliced (exon 2-containing) OAT mRNA, the filters that were previously used to examine the fractionation of the spliced OAT species (fig.13) were stripped and rehybridized with probe P2. Fig. 26 indicates that the unspliced mRNA is not efficiently translated in either strain, since it appears only in the light fractions of the gradient. Treatment with cycloheximide (fig. 27, RB355(+), Y79(+)) results in a minor shift in the gradient, as opposed to no treatment (fig.27 RB355(-), Y79(-)), a finding that is consistent with poor initiation.

#### C.3. Lack of effect of eIF-4E on translation of exon 2-containing OAT mRNA

After stripping, the filters used in fig.21 were rehybridized with P2 to determine if the levels of eIF-4E modulate the translational efficiency of the unspliced OAT transcript. Fig.28 indicates that the exon 2-containing OAT mRNA did not shift toward denser fractions in the gradient upon transfection by either eIF-4E or eIF-4E(A) . This

**Figure 26.** Polysome profile of unspliced OAT mRNA.

The filter from the experiment in fig. 13 was stripped and reprobbed with P2 to identify the exon 2-containing (unspliced) OAT mRNA (see Methods).

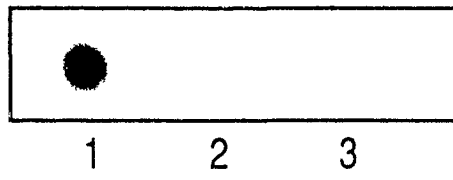
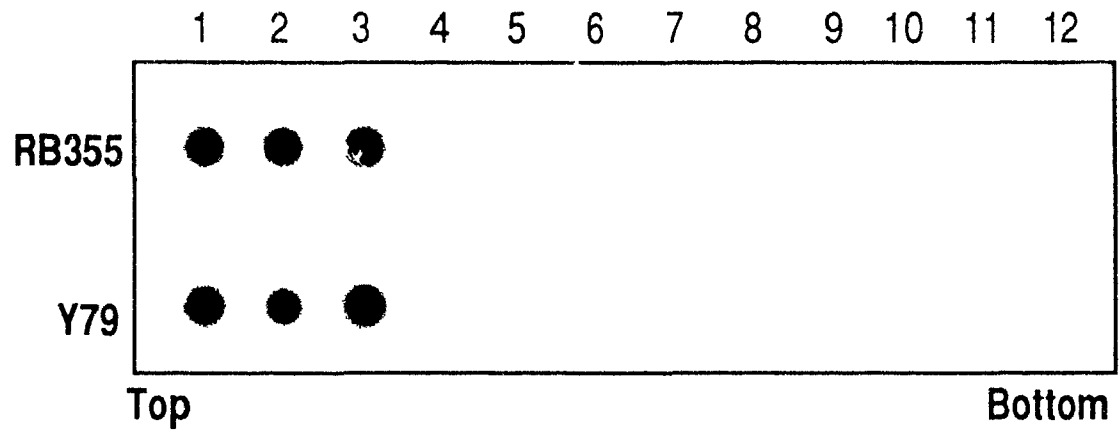


**Figure 27.** Polysome profile of unspliced OAT mRNA before and after cycloheximide treatment.

(-) Polysome profile of untreated RB355 and Y79 cells.  
(+) Polysome profile of RB355 and Y79 after treatment in culture with 0.75  $\mu$ g/ml cycloheximide for 30 mins. The filter from figure 16 was stripped and reprobed with P2 to identify the unspliced OAT mRNA.

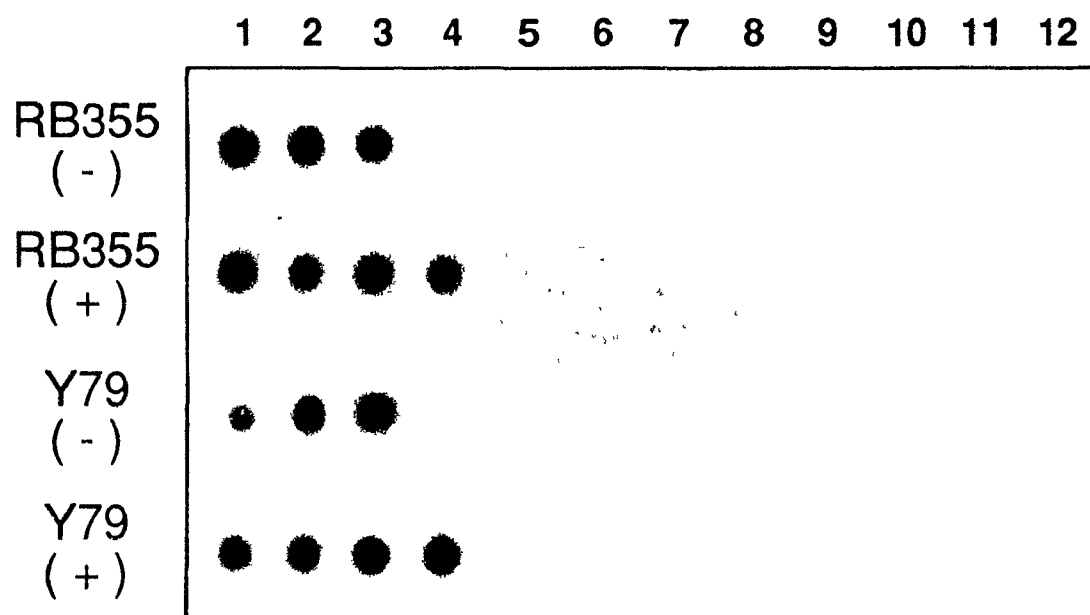
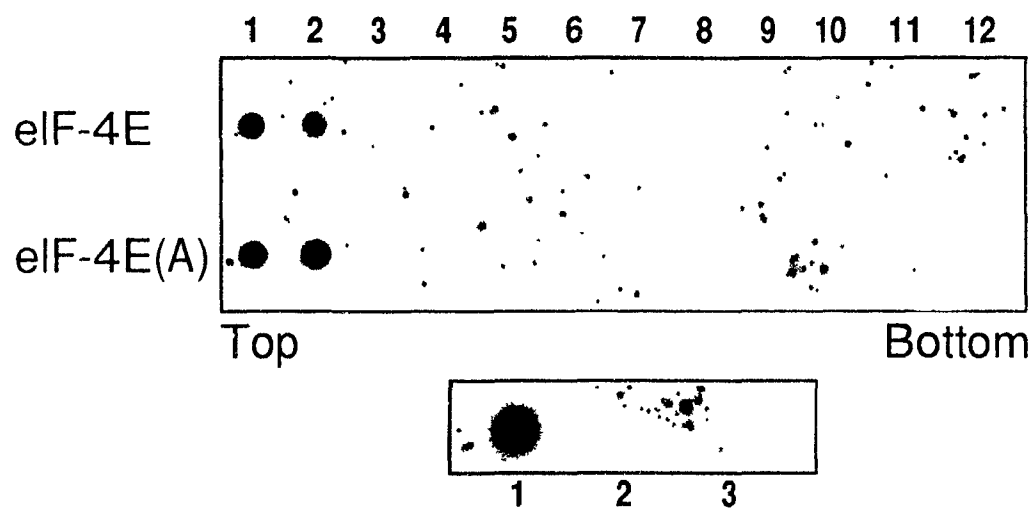


**Figure 28.** Polysome profile of unspliced OAT mRNA in RB355 transfectants.

The filter from the experiment in figure 21 was stripped and reprobbed with P2 to identify the exon 2-containing (unspliced) OAT mRNA.



observation suggests that the inefficient translation of the unspliced OAT mRNA is determined by an alternative factor(s) or mechanism(s).

#### **D. Hormonal Regulation of OAT in Retinoblastomas RB355 and Y79**

##### **D.1. Effect of estrogen and $T_3$ on OAT protein, activity, and mRNA levels**

Treatment of RB355 and Y79 with estradiol for 0,6,12 or 24 hrs resulted in a gradual increase in OAT protein and activity; Table 3 illustrates the results obtained for the 24-hour time point. In RB355, estradiol elicited a 4-fold increase in OAT protein levels (fig.29), with a corresponding increase in OAT enzyme activity (fig.30). This pattern of stimulation was similar in estradiol-treated Y79 cells; a 6-fold increase in OAT protein (fig.29) and enzyme activity (fig.30) was observed after 24 hrs.

Treatment with  $T_3$  (Table 3) for 24 hrs. produced a similar response in the two strains; OAT protein (fig.29) and enzyme activity (fig.30) increased approximately 5-fold in both strains. It is interesting to note that the strain differences in the levels of OAT protein and enzyme activity are maintained, even after hormonal treatment.

Northern analysis demonstrated a 3-4 fold increase in OAT mRNA in RB355 and Y79, after estradiol treatment (fig. 31;

**Table 3.** Effects of estradiol and thyroid hormone ( $T_3$ ) on OAT protein and enzyme activity.

RB355 and Y79 strains were treated for 0 and 24 hours with  $10^{-6}$  M estradiol or  $10^{-6}$  M  $T_3$ . Preparation of extracts, immunoblotting and activity measurements were performed as described in Methods. 40 and 80  $\mu$ g cell extract were used to assay immunoreactive protein and enzyme activity, respectively. The values for the estradiol experiments represent the mean  $\pm$  s.d. of 3 experiments. The data for  $T_3$  are values for 2 separate experiments.

| ESTRADIOL |                         |                 |                                      |               |
|-----------|-------------------------|-----------------|--------------------------------------|---------------|
|           | OAT PROTEIN<br>(% Area) |                 | OAT ACTIVITY<br>(nmol/hr/mg protein) |               |
|           | 0 hrs.                  | 24 hrs.         | 0 hrs.                               | 24 hrs.       |
| RB355     | 5.3 $\pm$ 0.2           | 19.7 $\pm$ 0.4  | 125 $\pm$ 4                          | 513 $\pm$ 10  |
| Y79       | 15.5 $\pm$ 0.4          | 103.0 $\pm$ 4.0 | 362 $\pm$ 6                          | 2054 $\pm$ 39 |
| -----     |                         |                 |                                      |               |
| $T_3$     |                         |                 |                                      |               |
|           | OAT PROTEIN<br>(% Area) |                 | OAT ACTIVITY<br>(nmol/hr/mg protein) |               |
|           | 0 hrs.                  | 24 hrs.         | 0 hrs.                               | 24 hrs.       |
| RB355     | 4.5/5.1                 | 18.7/19.9       | 105/106                              | 572/579       |
| Y79       | 17.4/17.0               | 87.4/89.7       | 329/336                              | 1530/1511     |

**Figure 29.** Effect of estradiol and  $T_3$  on OAT immunoreactive protein.

This figure is a graphic representation of the OAT protein data presented in Table 3.

## ESTRADIOL

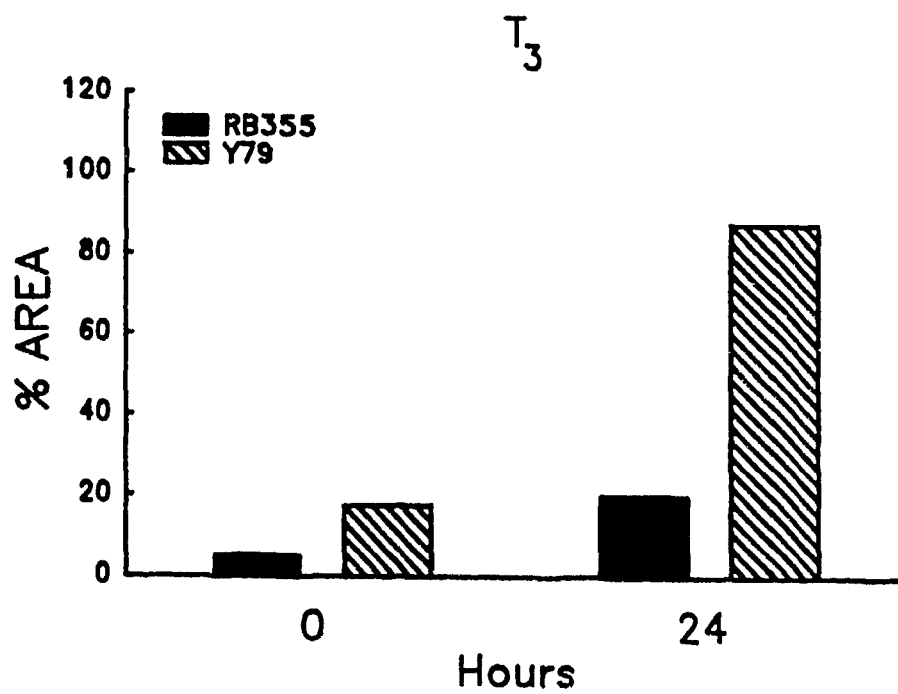
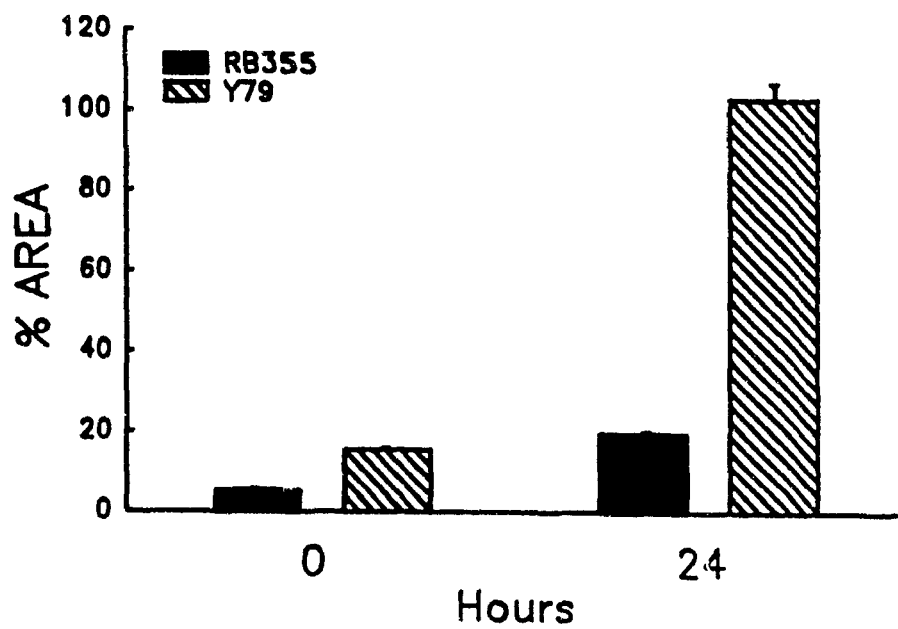


**Figure 30.** Effect of estradiol and  $T_3$  on OAT enzyme activity.

This figure is a graphic representation of the OAT enzyme activity data presented in Table 3.

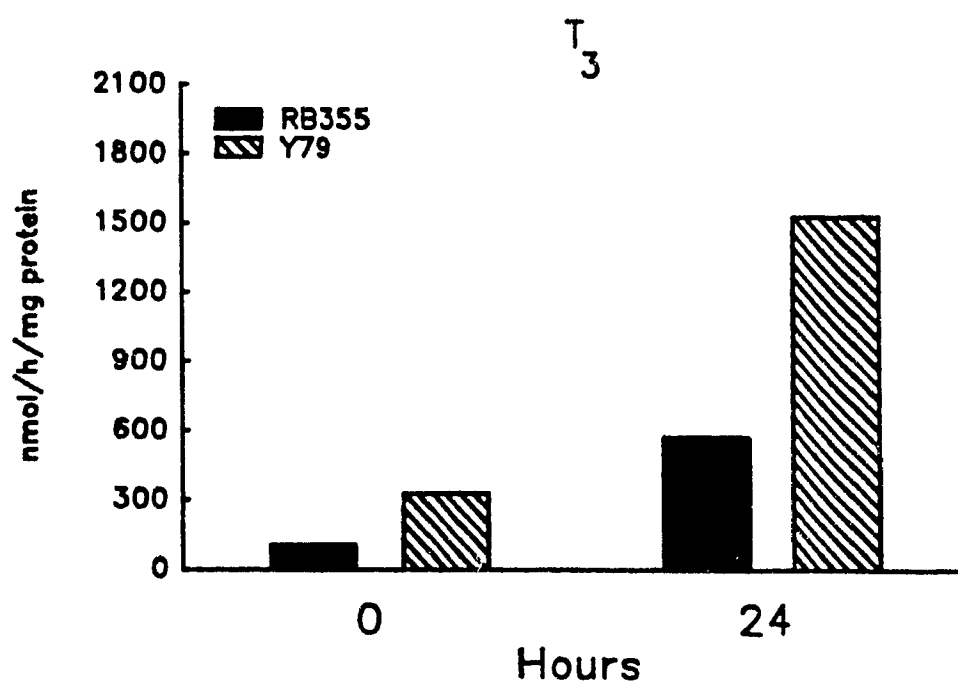
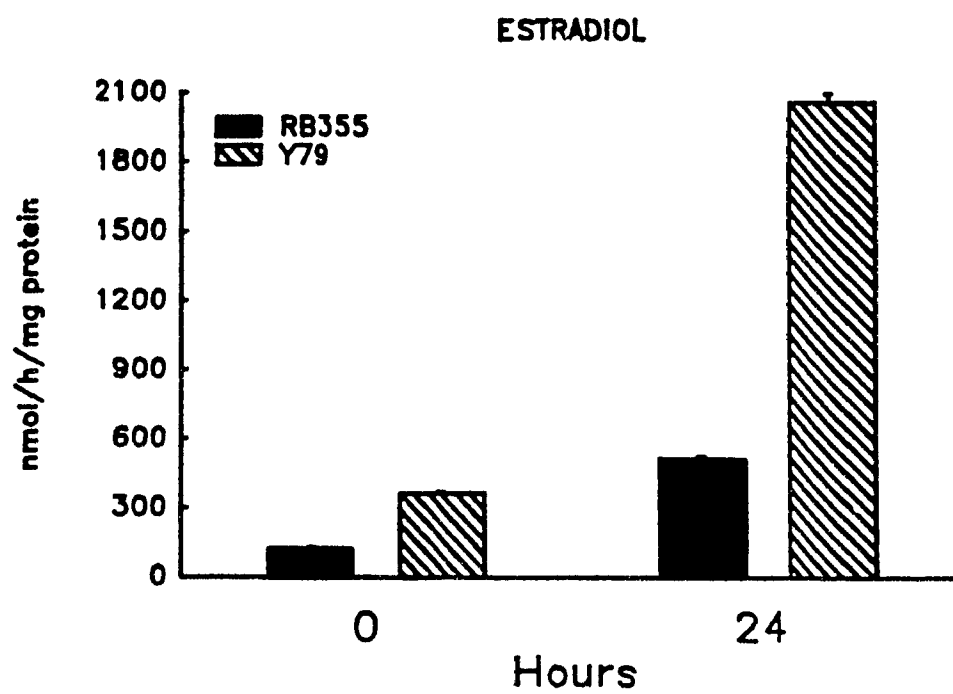
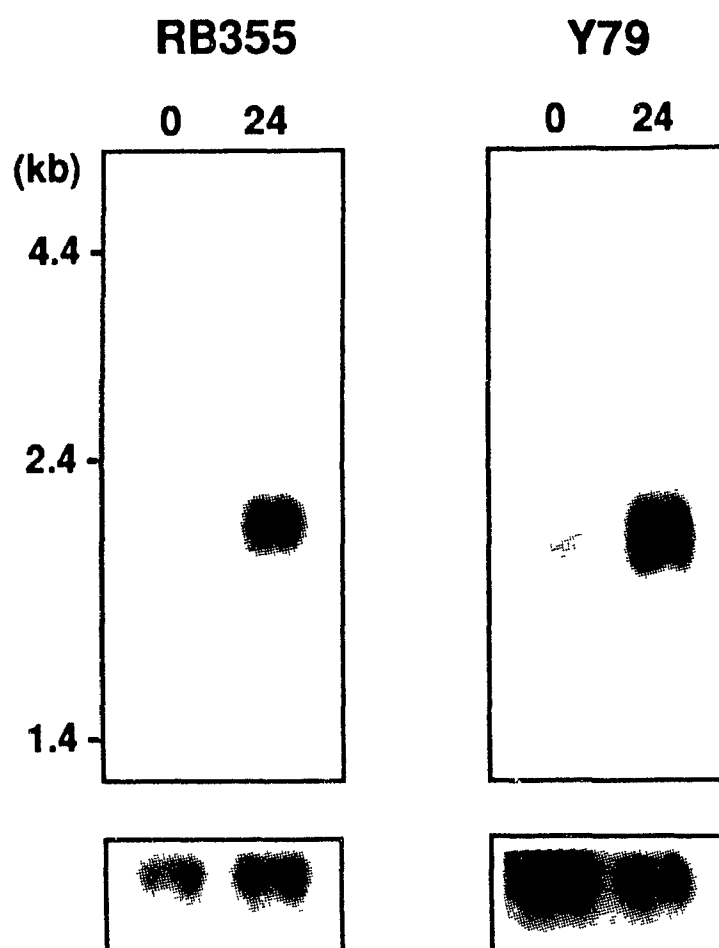


**Figure 31.** Northern analysis of RB355 and Y79 poly A<sup>+</sup> RNA after treatment with estradiol.

Retinoblastomas were treated with  $10^{-6}$  M estradiol for 0 and 24 hours as described in Methods. 10 $\mu$ g of poly A<sup>+</sup> RNA was electrophoresed on a 1.2% formaldehyde - agarose gel, transferred to a nitrocellulose membrane, and hybridized to an OAT cDNA (upper panels). The lower panels depict the same Northern blots, stripped and reprobed with the MTHFD cDNA as a control for the amount of RNA loaded per lane. Densitometric analysis of the ratios of OAT mRNA:MTHFD mRNA indicated a 3-4 fold increase after estradiol treatment in both strains.



result represents ratio of OAT mRNA:MTHFD mRNA). OAT mRNA levels in both strains were 5-7 fold greater after 24 hrs of  $T_3$  treatment (fig. 32).

#### D.2. Transcription of OAT in RB355 and Y79 after treatment with estrogen or $T_3$

The Northern analyses suggested that estradiol and  $T_3$  enhanced OAT synthesis by increasing transcription, since parallel increases were observed for OAT mRNA, protein, and enzyme activity in each strain. Nuclear run-on analyses were performed to determine whether an increase in transcription was indeed the mechanism of hormone action. Fig. 33 depicts the analyses performed on RB355 and Y79 in the presence of estrogen for 0,2,4, and 12 hours. The % stimulation for the time course was negligible. Transcription of the estrogen receptor gene was used as a positive control; a two-fold increase in transcription was observed and was verified by Northern analysis (data not shown). These findings suggest that estrogen affects OAT synthesis mainly at the post-transcriptional level in retinoblastomas.

Fig. 34 is a nuclear run-on analysis in the presence of  $T_3$ . The % stimulation over basal rates is at a maximum of 5-7-fold after 8 hours in both strains. It then decreases slightly at 12- 24 hours. Graphic representation of the run-on data has taken into account hybridization to the negative

**Figure 32.** Northern analysis of RB355 and Y79 poly A<sup>+</sup> RNA after treatment with T<sub>3</sub>.

Retinoblastomas were treated with  $10^{-6}$  M T<sub>3</sub> for 0 and 24 hours as described in Methods. 10 $\mu$ g of poly A<sup>+</sup> RNA was electrophoresed on a 1.2% formaldehyde - agarose gel, transferred to a nitrocellulose membrane, and hybridized to an OAT cDNA (upper panels). The lower panels depict the same Northern blots, stripped and reprobated with the MTHFD cDNA as a control for the amount of RNA loaded per lane. Densitometric analysis of the ratios of OAT mRNA:MTHFD mRNA indicated a 5-7 fold increase after T<sub>3</sub> treatment in both strains.

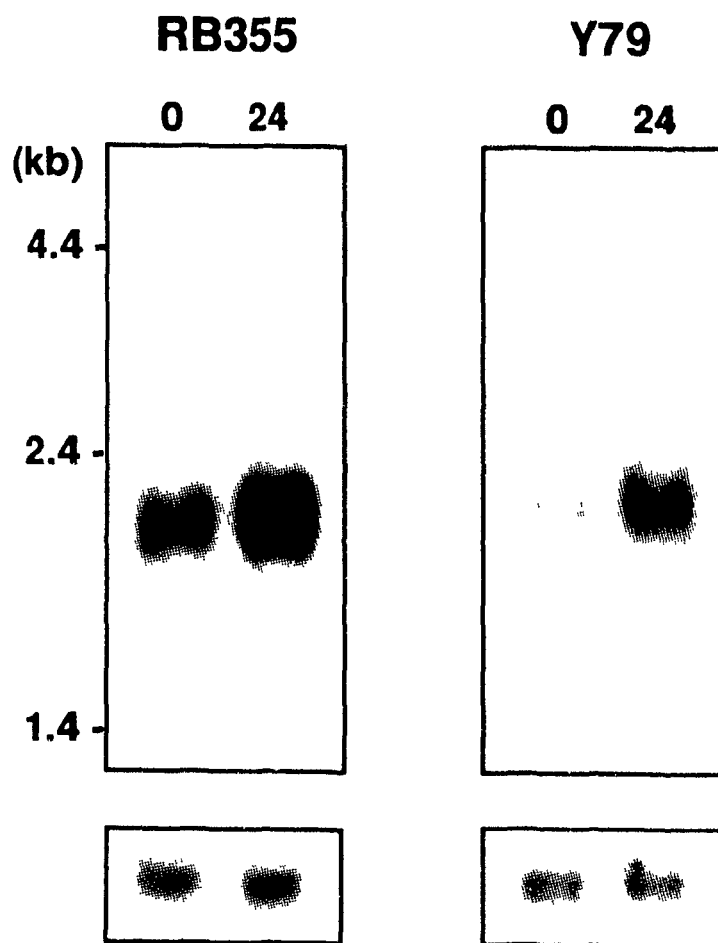


**Figure 33.** Nuclear run-on analysis of the OAT gene after estrogen treatment.

RB355 and Y79 cells were treated in culture with  $10^{-6}$ M estradiol for the indicated times, followed by isolation of nuclei. Plasmid DNA containing the OAT cDNA (50ng and 500ng) was digested with EcoRI and dot-blotted onto a nitrocellulose filter.  $^{32}$ P-labelled RNA synthesized by RB355 nuclei ( $2.0 \times 10^8$  cpm/time point; top panel) and by Y79 nuclei ( $2.0 \times 10^8$  cpm/time point; bottom panel) was hybridized to the membrane. EcoRI-linearized BlueScript vector was dotted onto the membranes as a control for non-specific hybridization (data not shown), as well as the cDNA for the human estrogen receptor, as a positive control (data not shown). The autoradiograms were scanned by laser densitometry, the negative control (vector) was subtracted, and the results are presented as a % of the zero hour time point.

## Estrogen

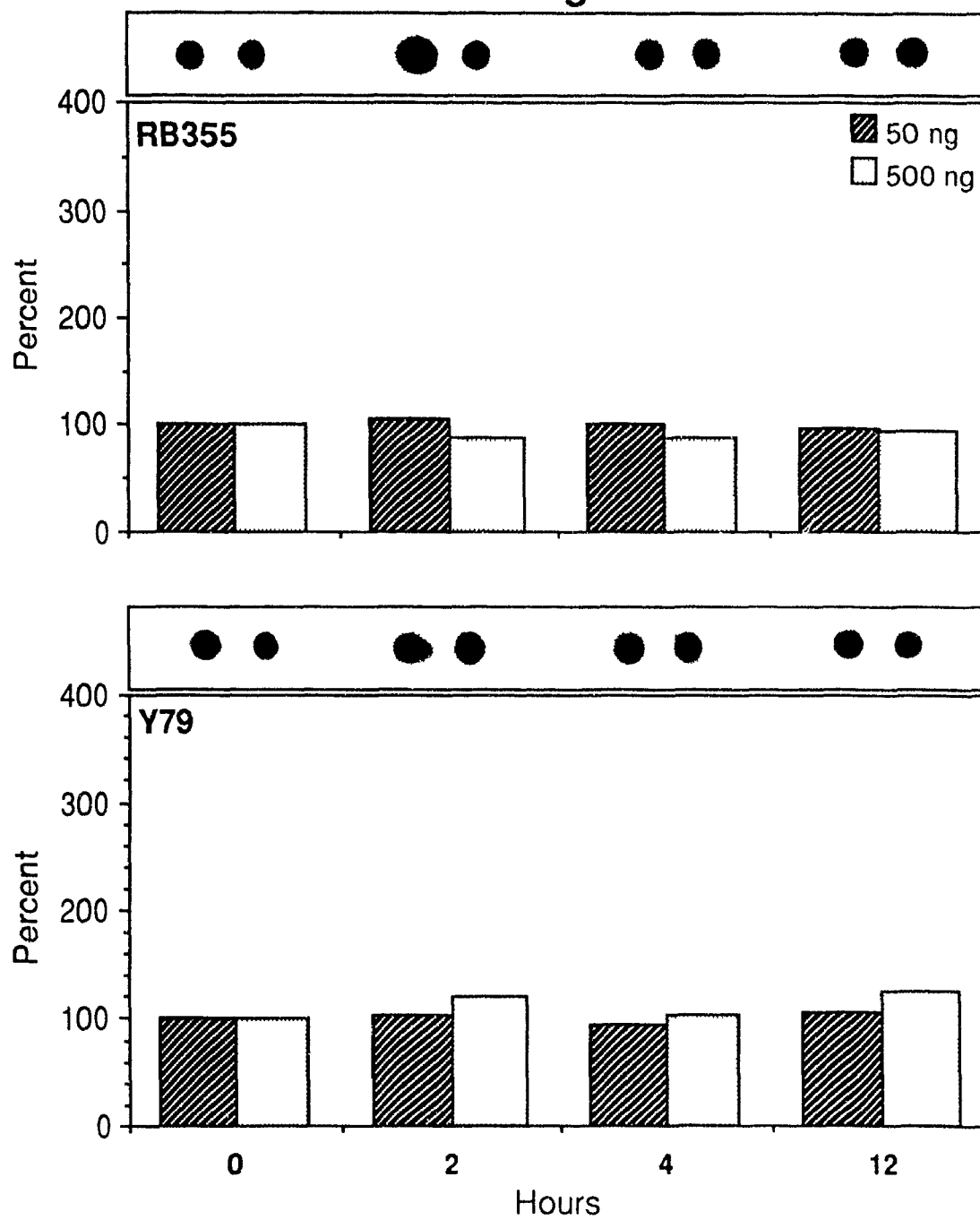
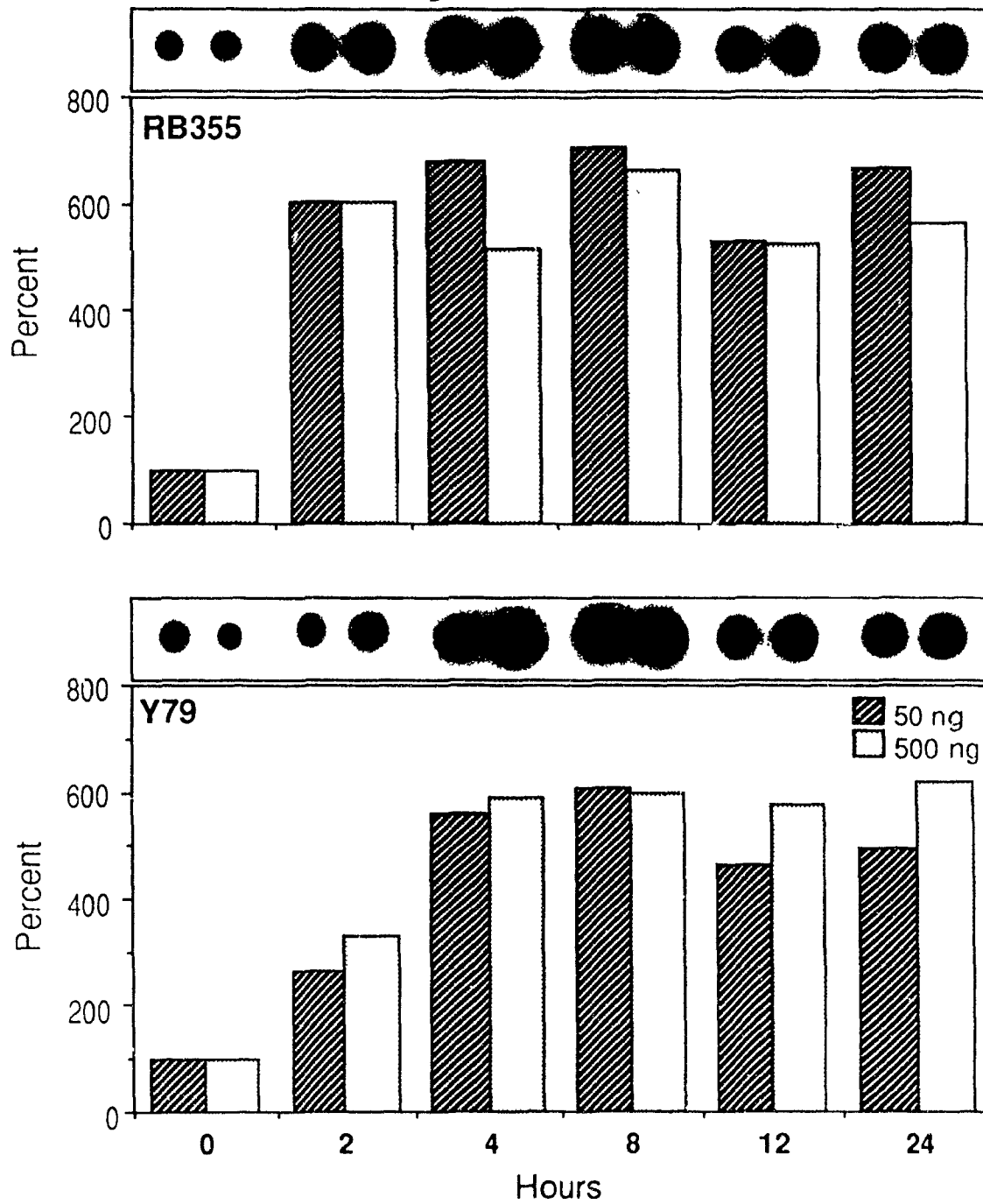


**Figure 34.** Nuclear run-on analysis of the OAT gene after  $T_3$  treatment.

RB355 and Y79 cells were treated in culture with  $10^{-6}M$   $T_3$  for the indicated times, followed by isolation of nuclei. Plasmid DNA containing the OAT cDNA (50ng and 500ng) was digested with EcoRI and dot-blotted onto a nitrocellulose filter.  $^{32}P$ -labelled RNA synthesized by RB355 nuclei ( $2.0 \times 10^8$  cpm/time point; top panel) and by Y79 nuclei ( $2.0 \times 10^8$  cpm/time point; bottom panel) was hybridized to the membrane. EcoRI-linearized BlueScript vector was dotted onto the membranes as a control for non-specific hybridization (data not shown). The autoradiograms were scanned by laser densitometry, the negative control (vector) was subtracted, and the results are presented as a % of the zero hour time point.

## Thyroid Hormone



control by subtracting each respective negative control value from its corresponding transcription value. This may result in some points where the graphic representation of the data may not seem like an accurate reflection of the intensity of the dot depicted above the graph.

The increase in transcription corroborates the 5-7-fold increase in mRNA observed on Northern analysis after treatment with  $T_3$ ; and indicates that this hormone acts mainly as a transcriptional activator of OAT in retinoblastomas.

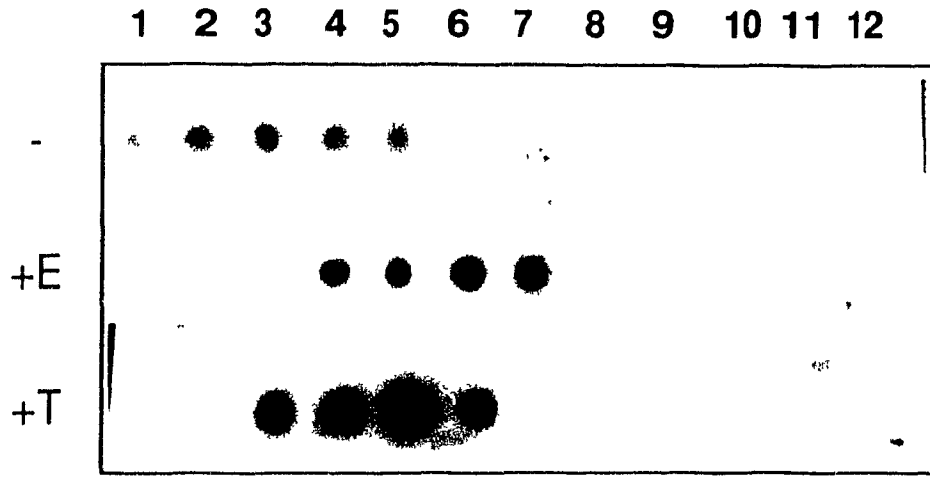
#### D.3. Polysome analysis of OAT mRNA after treatment with estrogen or $T_3$

A feasible post-transcriptional mechanism of action that estrogen might employ would be to increase the translation of the OAT mRNA. Increasing the stability of the OAT mRNA might lead to increased translation (Mullner et al 1989), or, conversely, increased initiation of translation might lead to increased stability of the OAT mRNA (Belasco et al 1986, Kelly et al 1987).

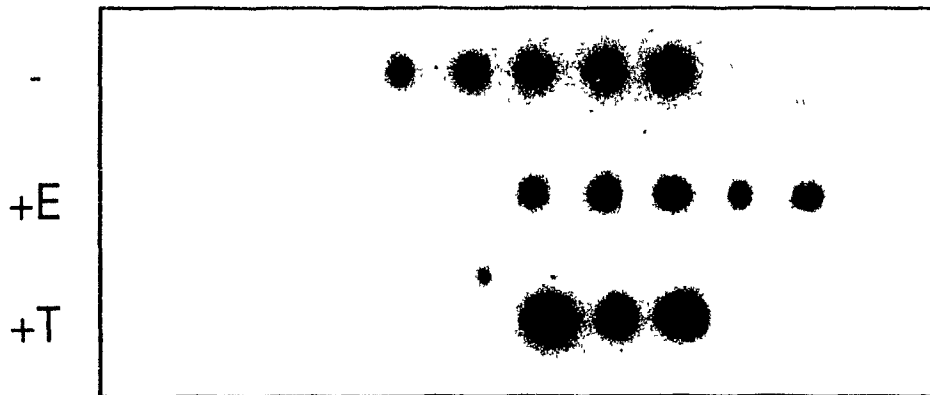
Polysomes were analyzed before (fig.35(-)) and after 24 hours of treatment with estrogen (fig.35, +E) and  $T_3$  (fig.35, +T), to determine whether the hormones caused increased loading of polysomes on the mRNA. In RB355 and Y79, estrogen caused a shift of the OAT mRNA into two additional polysome fractions: from fractions 1-5 to 4-7 in RB355, and from 5-9 to

**Figure 35.** Polysome profile of spliced OAT mRNA before and after treatment with estrogen or  $T_3$ .

(-) Polysome profile of untreated RB355 and Y79 cells. (+E), Polysome profile of RB355 and Y79 after treatment in culture with  $10^{-6}M$  estradiol for 24 hours. (+T), Polysome profile of RB355 and Y79 after treatment in culture with  $10^{-6}M$   $T_3$  for 24 hours. Fractionation, PCR, and dot blot analyses were performed as in figure 13. The filter was probed with P4 to identify the spliced OAT mRNA.



RB355



Y79

7-11 in Y79.  $T_3$  caused a shift into one additional fraction in RB355 (from 1-5 to 3-6), whereas in Y79 the OAT mRNA did not shift into additional denser fractions. The same experiment performed on MTHFR mRNA (fig.36) as a control, revealed no shift.

#### D.4. Effect of estrogen or $T_3$ on OAT translational initiation

If initiation is increased by estrogen or  $T_3$ , then the presence of cycloheximide together with hormone should cause an even greater shift of the OAT mRNA in the polysome gradient (see section A.4 for explanation of effects of cycloheximide). Fig. 37 (+C) is the profile of the spliced OAT mRNA in the presence of cycloheximide (fractions 3-7 for RB355, and 10-12 for Y79). Addition of estrogen prior to cycloheximide (fig.37 (+C+E)) caused a significant shift, up to fractions 6-9, in RB355. Since the OAT mRNA in Y79 cells after cycloheximide treatment is already in the densest fractions, no dramatic change was observed with estrogen.

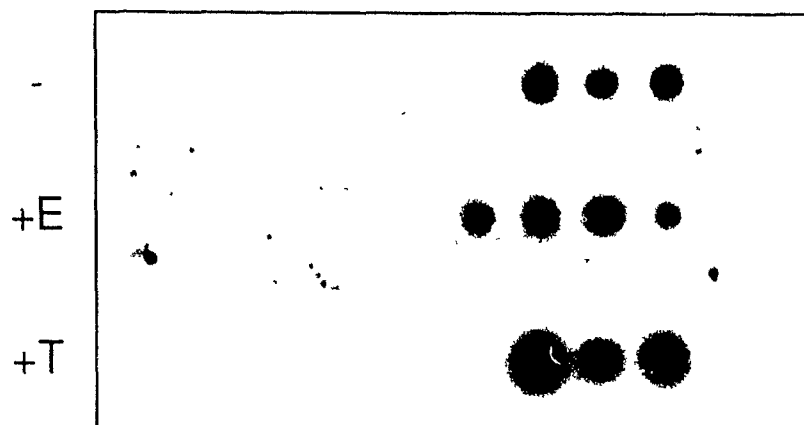
$T_3$  in concert with cycloheximide (fig.37 (+C+ $T_3$ )) has little or no effect; a shift to fractions 4-7 in RB355 and again, no significant change for Y79. MTHFR mRNA is observed at the bottom of the gradient, as previously observed, when the analysis was performed after cycloheximide treatment (refer to fig.17).

Therefore estradiol appears to act by increasing the rate

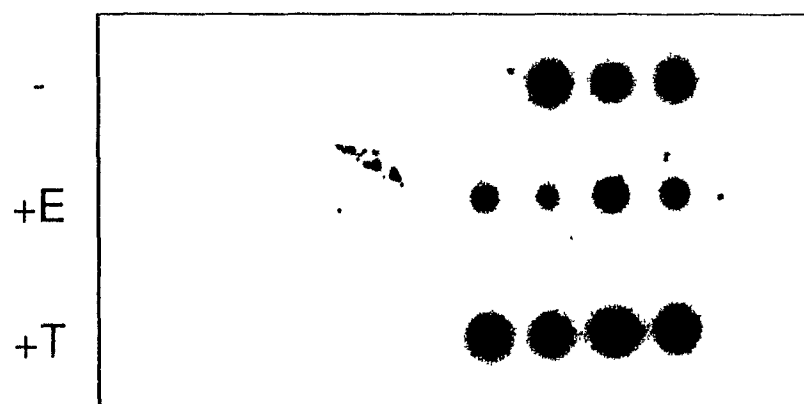
**Figure 36.** Polysome profile of MTHFR mRNA before and after treatment with estrogen or  $T_3$ .

(-) Polysome profile of untreated RB355 and Y79 cells. (+E), Polysome profile of RB355 and Y79 after treatment in culture with  $10^{-6}M$  estradiol for 24 hours. (+T), Polysome profile of RB355 and Y79 after treatment in culture with  $10^{-6}M$   $T_3$  for 24 hours. Fractionation, PCR, and dot blot analyses were performed as in figure 14. The filter was probed with an MTHFR-specific oligonucleotide to identify MTHFR mRNA.

1 2 3 4 5 6 7 8 9 10 11 12



RB355



Y79

**Figure 37.** Polysome profile of spliced OAT mRNA in the presence of cycloheximide before and after treatment with estrogen or  $T_3$ .

(+C) Polysome profile of RB355 and Y79 cells after treatment in culture with 0.75  $\mu\text{g/ml}$  cycloheximide for 30 mins. (+C+E) Polysome profile of RB355 and Y79 after treatment in culture with  $10^{-6}\text{M}$  estradiol for 24 hours and 0.75  $\mu\text{g/ml}$  cycloheximide for the final 30 mins. of the estradiol treatment. (+C+T) Polysome profile of RB355 and Y79 after treatment in culture with  $10^{-6}\text{M}$   $T_3$  for 24 hours and 0.75  $\mu\text{g/ml}$  cycloheximide for the final 30 mins. of the  $T_3$  treatment.

Fractionation, PCR, and dot blot analyses were performed as in fig. 13. The filter was probed with P4 to identify spliced OAT mRNA.



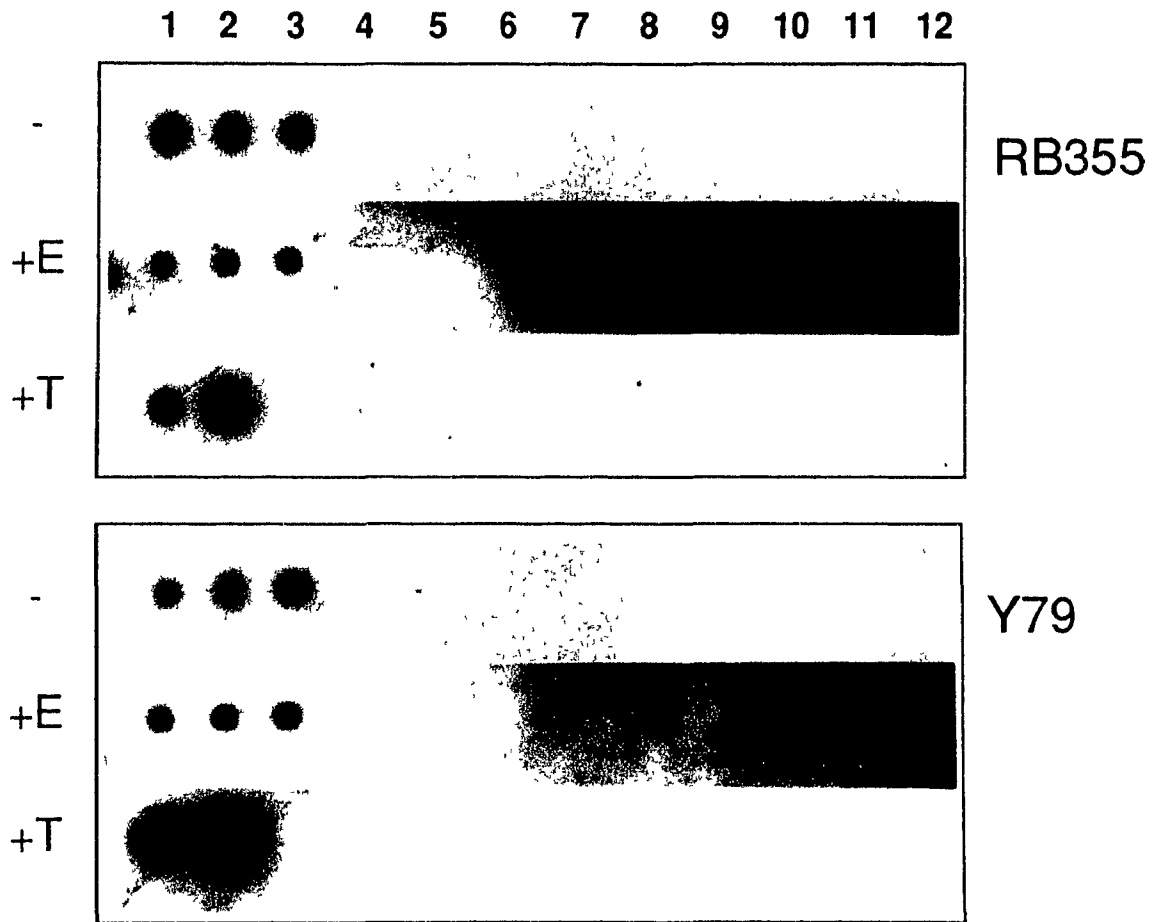
of translational initiation of the spliced OAT mRNA. The effect of  $T_3$ , if any, is much less pronounced.

The filters used in fig.35 and fig.37 were stripped and reprobed with P2, the exon 2-specific probe. No shift of the unspliced OAT mRNA was observed in the presence of estrogen or  $T_3$  (fig.38 (+E,+T) respectively).

Similar results were obtained when the unspliced OAT mRNA's polysome distribution was examined in the presence of cycloheximide and hormone. No shift was observed in either strain with cycloheximide plus estrogen or thyroid hormone when compared to cycloheximide alone (fractions 1-4 for (+C), (+C+E), (+C+T), data not shown).

**Figure 38.** Polysome profile of unspliced OAT mRNA before and after treatment with estrogen or  $T_3$ .

(-) Polysome profile of untreated RB355 and Y79 cells. (+E), Polysome profile of RB355 and Y79 after treatment in culture with  $10^{-6}M$  estradiol for 24 hours. (+T), Polysome profile of RB355 and Y79 after treatment in culture with  $10^{-6}M$   $T_3$  for 24 hours. The filter from fig. 35 was stripped and reprobbed with P2 to identify the unspliced OAT mRNA.



## V. DISCUSSION

### A. Modulation of OAT Translation by eIF-4E

In retinoblastomas RB355 and Y79, we detected the presence of a post - transcriptional mechanism for OAT regulation. Although both strains contain equal amounts of OAT mRNA, and synthesize the OAT mRNA at similar rates as shown by the nuclear run-on assays, Y79 expresses 3-fold more OAT protein and activity than RB355.

The polysome profiles support these observations since the spliced OAT mRNA appear in the lighter polysome fractions of the RB355 strain, as compared to Y79. This finding indicates that there are fewer ribosomes associated with this OAT mRNA in the RB355 strain and is consistent with reduced translation of OAT. The fact that the MTHFR mRNA appears in essentially the same polysome fractions for both cell lines suggests that the OAT translational difference is mRNA-specific, rather than a universal property of the strain. Our finding of similar immunoreactive actin levels in the strains is also compatible with OAT-specific differences in these retinoblastomas.

Mueckler et al (1983B) reported the presence of a translational and pretranslational control mechanism for OAT in rat liver. When rats were maintained on a 60% protein diet, as compared to 0%, OAT protein synthesis increased 100-

fold, while mRNA levels increased only 2-fold. The dramatic enhancement in protein synthesis was due to a 2-fold increase in the rate of elongation, as well as a 23-fold increase in the number of polysomes present on OAT transcripts of the rats fed the 60% protein diet. It is possible, therefore, that the for post-transcriptional regulation of OAT in retinoblastomas may occurs at either of these 2 levels: translational initiation or elongation of the polypeptide chain. To distinguish between the 2 possibilities, we used the protein synthesis inhibitor cycloheximide, which reduces the rate of elongation such that elongation, rather than initiation, becomes the rate-limiting step of translation (Fan et al 1970, Lodish 1971). The cycloheximide data suggest that the strains initiate OAT translation with different efficiencies, although these results alone would not rule out a difference in the rate of elongation, as well as in the rate of initiation. A similar line of investigation was used by Fan et al. (1970) to determine that a difference in the rate of protein synthesis in interphase vs. mitosis was due to a difference in the rate of initiation. As well, Lodish (1971) showed that a different rate of translational initiation was responsible for the levels of alpha and beta globin protein in mammalian reticulocytes.

The translational regulatory control observed for OAT in this study may relate to the fact that the two retinoblastomas are in different stages of differentiation; the Y79 strain

consistently had a higher growth rate than RB355 (R. Fagan, personal observation). The availability of specific translation factor(s), and/or the state of such factor(s), in the RB355 strain may be different from that in the Y79 strain. In light of the cycloheximide data, these factors presumably would be involved in initiation of translation.

Eukaryotic initiation factor eIF-4E is found in limiting amounts in the cell, relative to other factors, and thus a potential candidate for translational control (Hiremath et al 1985, Duncan et al 1987, Hiremath et al 1989). The active form of eIF-4E has been shown to be phosphorylated on one major site, serine codon 53 (Rychlik et al 1987). The proposed kinase/phosphatase enzymes that control the level of phosphorylation are presumed to be present in non-rate-limiting amounts (Lazaris-Karatzas et al 1990, De Benedetti et al 1990). The levels of eIF-4E mRNA and immunoreactive protein are lower in the RB355 strain, relative to the Y79. Overexpression of a wild-type eIF-4E cDNA in the RB355 strain caused a shift of the OAT mRNA toward the denser fractions of the gradient, those fractions normally observed for the OAT mRNA in the Y79 strain. A concomitant increase in the amount of OAT immunoreactive protein, to the level normally seen in Y79, was also observed. These results allow us to conclude that the decreased efficiency of initiation of the OAT spliced mRNA in RB355 is due to a limiting amount of eIF-4E. The OAT mRNA may not be a good competitor for this protein, relative

to other cellular transcripts such as MTHFR, and overexpression of eIF-4E alleviates the translational repression. In addition, the finding that overexpression of an initiation factor restores OAT abundance to that observed in Y79, argues against a difference in the rate of elongation between the strains.

Our experiments indicate that cellular levels of eIF-4E can modulate OAT translation. Despite the enhanced translation with eIF-4E overexpression, the polysome profile for OAT mRNA and MTHFR mRNA suggests that OAT is not translated as well as MTHFR. MTHFR translation is not subject to regulation by increasing eIF-4E levels. This suggests that other factors may be involved in the regulation of translation of these mRNAs.

The structural characteristics in the 5' UTR of OAT that may render it less competitive for eIF-4E, as compared to other cellular mRNAs, are not known. Steric hindrance of the 5' cap (Godefroy-Coburn et al 1985), and 5' proximal secondary structure (Pelletier et al 1985) are features suggested to play a role in translational efficiency of mRNAs, as modulated by eIF-4E. A current model (Sonenberg 1988) suggests that eIF-4E is important in bringing eIF-4A to the 5' end of the mRNA. eIF-4A then destabilizes secondary structure (Rozen et al 1990), which facilitates the subsequent binding and scanning of the 43S preinitiation complex toward the initiation codon (Sonenberg et al 1988). This model predicts

an order of translatability of mRNAs, which relates to the need for increased levels of eIF-4E. Increasing secondary structure in the 5' UTR, and/or increasing inaccessibility of the cap is inversely proportional to the translational efficiency of that mRNA. In order to improve the efficiency of translation, the levels of eIF-4E must be increased.

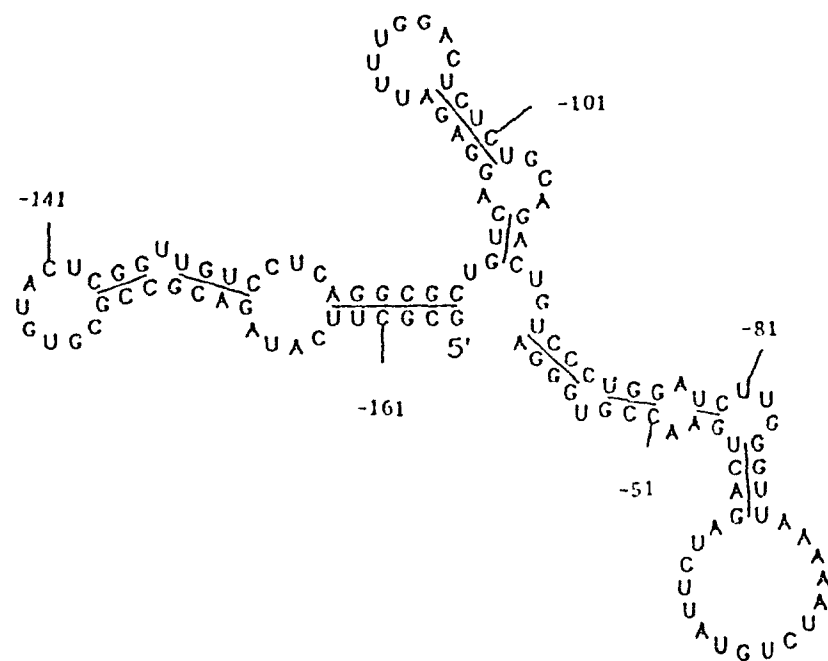
RNA secondary structure analysis of OAT exon 1 predicted an overall  $\Delta G^\circ = (-)16.9$  kcal/mole using Turner's rules (fig.39), with an involvement of the nucleotides very close to the 5' cap in a secondary structure. Whether the predicted secondary structure is sufficiently stable and/or the cap region sterically hindered to the point of causing OAT to be less competitive for eIF-4E remains to be tested.

Exon 1 has been implicated elsewhere to play a role in the regulation of OAT expression. In the course of analyzing the human OAT gene promoter, Engelhardt et al. (1991) noted that exon 1 was necessary to achieve maximal expression of their constructs. This was true for both liver and kidney derived cell lines, but the exact role of the exon, whether it functioned at the level of transcription, mRNA stability, or translation, remains to be examined.

Overexpression of eIF-4E in NIH3T3 and Rat 2 fibroblasts (Lazaris-Karatzas et al 1990), or in HeLa cells (De Benedetti et al 1990) results in their tumorigenic transformation. Modulation of translation of certain oncogenes or growth factors has been postulated as the mechanism of action of eIF-

**Figure 39.** Secondary structure analysis of the OAT mRNA 5' UTR (spliced and unspliced).

Secondary structure of OAT mRNA with an unusual folding region, predicted by Monte Carlo simulation, (-)161 to (-)44. The lowest free energy of the spliced OAT and unspliced OAT were calculated using Turner's rules and are -16.9 kcal/mole and -23.8 kcal/mole respectively. The analysis was kindly performed by Dr. Shu-yun Le, National Research Council, Ottawa, Ontario).



4E, a putative proto-oncogene (Lazaris-Karatzas et al 1990, De Benedetti et al 1990). Kaspar et al. showed that eIF-4E phosphorylation (and consequently its activation) occurred simultaneously with recruitment of ribosomal protein L32 mRNA from ribonucleoprotein particles into polysomes (Kaspar et al 1990). Our data demonstrate a direct effect of eIF-4E on the translational initiation of a specific cellular transcript; this transcript was not associated with ribonucleoprotein particles since EDTA treatment shifted the OAT mRNA to the top of the gradient.

Overexpression of the mutated eIF-4E(A) in NIH-3T3 cells extended the doubling time for the strain, possibly due to a transdominant negative effect on growth (Lazaris-Karatzas et al 1990). A similar dominant negative effect may have occurred in our experiments since overexpression of eIF-4E (A) in RB355 appeared to shift the OAT mRNA to lighter fractions. The eIF-4E(A) may bind the 7-methyl-guanosine cap, and, in doing so, interfere with eIF-4E's ability to interact with the cap of the cellular mRNAs.

Translational regulation of OAT has been reported. High dietary protein and cAMP increase OAT protein synthesis in rat liver by increasing the rate of initiation; the mechanism was not determined (Merrill et al 1985A). cAMP is also a well-known modulator of cellular differentiation and may directly or indirectly affect the phosphorylation state and/or levels of eIF-4E. It is not clear why retinoblastomas RB355 and Y79

contain different amounts of eIF-4E although Y79 appears to have a faster growth rate and may be in a more undifferentiated state. There is no known role for OAT in the differentiation process but its modulation by a putative proto-oncogene alludes to an interesting function for this "house-keeping" enzyme.

#### **B. Exon-2 Containing OAT mRNAs-What are they doing?**

The finding of exon 2 in a subpopulation of the retinoblastoma OAT transcripts is a novel one, although its function remains to be elucidated. The exon 2-containing OAT mRNA is not efficiently translated in either the RB355 or Y79 strain. The cycloheximide data suggest that the unspliced mRNA does not initiate very efficiently, although we can not rule out a decreased rate of elongation also. As overexpression of eIF-4E has no effect on this mRNA, other post-transcriptional mechanisms are implied in regulation of OAT expression.

Therefore inclusion of the 87 nucleotides of exon 2 in the OAT mRNA decreases the translatability of the message. As no other AUGs exist, or other possible initiation codons in a favorable context (Kozak 1987), it is likely that the protein made from this mRNA initiates at the same position as its spliced counterpart (Mitchell et al 1988).

Exon 2 occupies a region of the 5' untranslated region of

the OAT mRNA, and, as such, may be involved in the regulation of translation. Its presence extends the length of the 5' UTR by 87 nucleotides; there is some evidence for decreasing translational efficiency as the length of the 5' UTR increases (Sonenberg 1988, Waterhouse et al 1990). The longer the 5' UTR, the greater the potential for secondary structure, and increased secondary structure has been shown to decrease translational efficiency (Pelletier et al 1985, Hershey et al 1986, Sonenberg 1988). The presence of exon 2 was predicted to increase the  $\Delta G^\circ$  of the 5' UTR from  $(-)$ 16.9 kcal/mole to  $(-)$ 23.8 kcal/mole as predicted by Turner's rules (fig.39). It is also possible that a protein binds to a sequence in exon 2, impeding the migration of the ribosomes down the message; this type of control has been seen for the 5' UTR of the ferritin gene (Caughman et al 1988).

If exon 2 played a critical role in the regulation of OAT, whether it be translational or not, one might expect to find it conserved in other tissues and species. To date it has not been detected in any other human tissues, *S. cerevisiae* and rat, the two other species in which OAT has been cloned (Mueckler et al 1985, Degols et al 1987). Exon 2 was initially observed in 2 processed OAT pseudogenes, located on the X chromosome of man (Mitchell et al 1988). Processed pseudogenes most likely arise from reverse transcription of a mRNA followed by integration into genomic DNA. This implies that at some time during evolution, exon 2-containing OAT

transcripts may have been more prevalent in the cells of humans or other species.

It is interesting to note that another gene, the urea cycle enzyme argininosuccinate synthetase (AS), undergoes a similar 5' alternative splice (Freitag et al 1984). AS mRNA is structurally arranged in a similar manner to OAT; the first two exons encode the 5'UTR, and translational initiation is in exon 3. In human cells, exon 1 and 3 are joined, deleting exon 2 from most of the AS mRNA. In contrast, the second exon is retained in most of the AS mRNA found in baboon cells (Freitag et al 1984). The function, if any, of AS exon 2 in human cells, as with OAT exon 2, has not yet been determined.

### **C. Transcriptional and Post-Transcriptional Effects of Estrogen and Thyroid Hormone on OAT**

Estrogen and thyroid hormone stimulated OAT expression in both retinoblastoma cell lines. In each case, the increases in OAT protein and activity paralleled the increases in mRNA levels after hormone treatment. To evaluate the mechanism(s) of hormonal action, transcriptional rates were measured by nuclear run-on analysis.

Increases in the rate of OAT transcription in the presence of estrogen were not significant in either strain, although, as a control, a 2-fold increase in the rate of estrogen receptor mRNA synthesis was observed. Conversely,

thyroid hormone elicited a 5-7-fold stimulation in the rate of OAT transcription in RB355 and Y79.

Therefore, estrogen and thyroid hormone's primary mechanism of activation of OAT expression apparently take place on 2 different levels: estrogen does not dramatically affect the rate of transcription whereas thyroid hormone does. Although both hormones were previously reported to be transcriptional activators of OAT in rat kidney, the full effect elicited by estrogen was not accounted for by transcriptional enhancement alone (Mueckler et al 1983A, Mueckler et al 1984). Whether or not this was an artifact of their system is unknown, and, as such, estrogen may or may not play a role in the post-transcriptional regulation of OAT in rat kidney.

Estrogen and thyroid hormone may increase translational initiation of the spliced OAT mRNA. In the presence of estrogen, the fractional distribution of these mRNA increased 2 fractions, indicating an increased number of polysomes. Thyroid hormone elicited a similar response, but to a much lesser degree. This experiment, performed in the presence of cycloheximide, revealed that the rate of initiation of translation of the spliced OAT transcript is increased in the presence of the hormones. Again a much greater effect was observed for estrogen, as opposed to thyroid hormone, indicating that estrogen has a greater effect on translational initiation. This was to be expected on the basis of the

transcriptional analysis of the OAT gene in the presence of the hormones. Since a 5-7-fold increase in OAT mRNA protein and enzyme activity was observed upon 24 hours of  $T_3$  treatment, and a similar increase in the rate of OAT transcription was observed after 8 hours of  $T_3$  treatment, a further increase in the rate of translational initiation seems superfluous.

Polysome analysis was performed after 24 hours of hormonal treatment. The transcriptional effect of the thyroid hormone may diminish, as indicated by nuclear run-on analysis, and the translational effect may increase at this time. In addition, the nuclear run-on autoradiogram may have been slightly over-exposed, and possibly no longer in the linear sensitive range. This would result in an overestimation of the actual  $T_3$  transcriptional activation for some time points. Furthermore, since the polysome data is not quantitative, the actual amount of mRNA involved in the shifted part of the gradient is unknown, and may represent a very small fraction of the total amount of OAT mRNA. In any event, it is clear that the primary effect of  $T_3$  on OAT synthesis is a transcriptional one; the translational effects may be very slight and may only occur secondarily to transcriptional activation.

As alluded to in the introduction, the post-transcriptional effects of estrogen on gene expression are varied. Among others, its effects include an increase in the

rate of initiation of translation of all mRNA species in the chick oviduct (Palmitter 1972, Pennequin et al 1978), an increase in the rate of peptide elongation on uterine ribosomes (Labate et al 1986), and increased stability of vitellogenin and ovalbumin mRNAs from chick liver (Gordon et al 1988), and oviduct (Palmitter et al 1974) respectively.

The mechanism for the estrogen-induced stimulation of initiation is not known, but it is conceivable that it affects the level/activity of any one of the initiation factors, which would then lead to an increase in initiation. Increasing the rate of initiation events would presumably lead to a greater density of ribosomes on the message at any one time. It has been hypothesized that ribosomes can shield mRNAs from endonucleolytic cleavage (Belasco et al 1986, Kelly et al 1987, Atwater et al 1987); if the hypothesis is true, this would lead to stabilization of the mRNA, hence increasing the absolute levels of the mRNA observed upon estrogen treatment.

The 3-fold difference in OAT activity between strains is maintained throughout the hormonal experiments. This suggests that the post-transcriptional response to estrogen and thyroid hormone does not involve eIF-4E, but some other event in the initiation of translation. The exact post-transcriptional mechanism still remains to be worked out.

In contrast to their effects on the spliced OAT mRNA, these hormones have no effect on translation of the unspliced mRNA. Cycloheximide treatment alone suggested that the

unspliced mRNA was initiating poorly; only a slight shift in the polysome gradients was observed. The same experiment performed in conjunction with hormonal treatment revealed no shift in either strain over that of the cycloheximide alone.

No change in the translation of the unspliced mRNA is observed with estrogen, even though it appears to increase initiation of translation of the spliced OAT mRNA. If poor initiation was the single factor causing the translational inefficiency of the unspliced OAT mRNA, then estrogen treatment in conjunction with cycloheximide would presumably cause a shift in the polysome distribution. It is possible that a greater shift is not observed because there is a block to the migration of the ribosomes down the unspliced mRNA. Estrogen in conjunction with cycloheximide would not cause a greater shift than cycloheximide alone as the mRNA, due to the block, would already be saturated with polysomes. A shift is not observed upon estrogen treatment alone due to the fact that the rate of elongation has to be slowed in order to observe polysome loading.

Therefore poor initiation and a block to elongating ribosomes may account for the translational inefficiency of the unspliced OAT mRNA. The mechanism may involve 5' cap inaccessibility, increased secondary structure, and/or a protein that binds a sequence or structure in exon-2, thereby impeding the migration of ribosomes.

## VI. SUMMARY

OAT is expressed to some extent in most tissues, and its expression can be controlled by a variety of hormonal and dietary stimuli in a subset of these tissues. Interestingly, a generalized deficiency of OAT in man manifests itself phenotypically in only one tissue, that being retina.

Characterization of the regulation of OAT in the retinally-derived cell lines, retinoblastomas RB355 and Y79, lead to several interesting conclusions, but no direct correlations were drawn regarding retinal susceptibility to OAT deficiency.

In RB355 and Y79, OAT is regulated at both the transcriptional and translational level. The amount of eucaryotic translation initiation factor eIF-4E can modulate the translational efficiency of the OAT mRNA. Estrogen also increases translational initiation of the OAT mRNA, without affecting transcription. Conversely, thyroid hormone is primarily a transcriptional regulator of OAT biosynthesis in the retinoblastomas.

A subset of the OAT mRNA in RB355 and Y79 was found to contain exon 2. This unspliced OAT mRNA was translationally inefficient in both strains, which was a phenomenon that was not affected by eIF-4E, estrogen or thyroid hormone.

## VII. CLAIMS to ORIGINALITY

- 1) This thesis represents the first comprehensive examination of the regulation of OAT in retinally-derived tissues.
- 2) I documented the post-transcriptional regulation of OAT in retinoblastomas (Fagan et al., 1989).
- 3) I demonstrated, through polysome analysis, that the post-transcriptional regulation of OAT in retinoblastomas was due to differences in translation of the OAT mRNA (Fagan et al., 1991).
- 4) To my knowledge, I am the first person to use PCR and dot-blot analysis in a polysome fractionation procedure (Fagan et al., 1991).
- 5) I demonstrated that the basis for the translational differences observed with the OAT mRNA was due to differences in the amount of translation initiation factor eIF-4E in the two strains (Fagan et al., 1991).
- 6) I am the first person to demonstrate that the amount of eIF-4E is able to modulate expression of a specific cellular message (Fagan et al., 1991).
- 7) I am the first person to demonstrate alternative splicing of OAT mRNA, i.e. the expression of the exon 2-containing OAT mRNA (Fagan et al., 1989).
- 8) I described the translational inefficiency of this mRNA, as compared to its spliced counterpart, in RB355 and Y79

(Fagan et al., 1991).

- 9) I documented the effects of estrogen and thyroid hormone on OAT mRNA, immunoprecipitable protein, and enzyme activity levels in the retinoblastomas (Fagan et al., 1989).
- 10) I demonstrated that the estrogen effect on OAT synthesis in the retinoblastomas is mainly post-transcriptional, an increase in translational initiation of the spliced OAT mRNA.
- 11) I demonstrated that thyroid hormone is primarily a transcriptional activator of OAT in retinoblastomas.
- 12) I demonstrated that estrogen and thyroid hormone have no effect on the translational efficiency of the unspliced OAT mRNA.

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