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Hormonal Regulation of Rat α_{2u}-Globulin Genes

by

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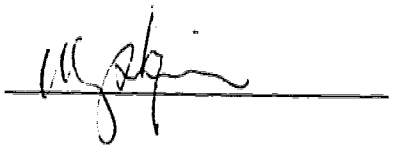

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List of Symbols, Nomenclature or Abbreviations

Dex	dexamethasone
Es	estrodiol
bp	basepair
kb	kilobasepair
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
ml	milliliter
µl	microliter
µg	microgram
mM	millimole
µM	micromole

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Abstract

α_{2u}-Globulin is a major urinary protein in male adult rats which functions as a carrier of pheromones. This protein is encoded by a multigene family of about 30 members. The expression of this gene is tissue specific and under multi-hormonal and developmental control. In male rat liver, which is the major source of urinary secreted α_{2u}-globulin, glucocorticoids induce the expression of this gene, as do androgens and growth hormone. Estrogen has a complex effect on the expression of this gene: it suppresses the expression of α_{2u}-globulin in male livers but in the liver of castrated females it initially induces, and then upon continued treatment, suppresses the expression of this gene.

This study was conducted with a particular member of the α_{2u}-globulin gene family, clone 91, transfected into mouse L-cells. The 5'-upstream sequences required to maintain a high level of induction by the glucocorticoid, dexamethasone, were defined through deletion analysis. Sequence between -762 bp and -226 bp was shown to be essential for this induction. Sequencing of the region revealed the presence of a glucocorticoid receptor binding site (GRE). Although the induction of α_{2u}-globulin gene by glucocorticoids is a secondary response, a requirement for direct binding of the activated glucocorticoid receptor was indicated. Clone #2, which has a base substitution at this GRE site loses inducibility by glucocorticoids. These observations suggest that the

expression of the clone 91 gene requires both the binding of activated glucocorticoid receptor and other regulatory factors induced by glucocorticoid.

In mouse L-cells expressing the human estrogen receptor, clone 91 was shown to be inducible by estrogen, which is similar to the initial response in the liver of castrated females upon estrogen treatment. This induction was dosage dependent and was inhibited by cycloheximide. The sequences responsible for this induction were, like those responsible for dexamethasone induction, located between -762 bp and -226 bp in the 5'-upstream region. An estrogen receptor element (ERE) is located in this region and it is conserved among most of the sequenced members of the gene family, indicating that most of the family members are subjected to estrogen regulation. Administration of both dexamethasone and estrogen produced a synergistic effect from which an interaction between the two receptors or other transcription factors they that they recruited can be inferred.

RT-PCR analysis of the α_{2u}-globulin RNA in different tissues of rats revealed that the regulation of the expression of this gene family is very complicated. The tissue specific, developmentally and hormonally regulated expression of α_{2u}-globulin appears to be due to differential regulation of individual genes or small group of genes. Our results suggest that the differentiated regulation of individual members is caused by a combination of differences in their regulatory sequences as suggested by the regulation of clones 91 and #2 in mouse L-cells, and tissue specific transcription factors.

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Introduction

Steroid hormone receptors are important transcription regulators that control gene expression in many organisms. These receptors regulate gene expression by interacting directly with the transcription initiation complex or via other transcription factors, and thereby stimulate or suppress the transcription of genes under their control (reviewed by Tsai and O'Malley 1994; Beato et al., 1995). The structure of steroid hormone receptors contains several functional domains, a N-terminal transactivation domain (AF-1), a dimerization domain (D), a DNA binding domain (DBD) and a transactivation domain (AF-2) located in the C-terminal region. Upon activation by hormones and binding to the recognition site on DNA, the transactivation domains activate target genes presumably by interacting with components of the core transcription machinery, coactivators, or other transcription factors. Several steroid hormone receptors have been demonstrated to activate transcription by enhancing the formation of stable preinitiation complexes at their target promotors, for example the AF-1 domain of thyroid receptor interacts specifically with TFIIB and the binding of TFIIB to the TFIID-DNA complex is one of the rate limiting steps in the formation of the preinitiation complex (Baniahmad et al., 1993).

The responses of genes to hormone action are classified as primary responses or secondary responses based on the time lag of response and whether or not protein synthesis is required. In primary responses, hormone-activated receptors bind productively to the primary-response genes and rapidly stimulate or repress their expression. This hormonally regulated gene expression is neither substantially delayed nor blocked by protein synthesis inhibitors (Godowski and Picard 1989). The DNA sequences of many primary response genes conform to a specific consensus that selectively binds hormone receptors *in vitro* and acts as a hormone response element (HRE) or suppresser *in vivo* (Yamamoto 1985; Mangelsdorf et al., 1995). The

common feature of the secondary response genes is that their hormonal regulation is preceded by a time lag and is blocked by protein synthesis inhibitors. It is believed that a transcription factor(s) is induced by hormones during this lag time, and this induced transcription factor alters the expression of secondary response genes. The mechanisms of both primary and secondary responses are still under investigation. Particularly in the secondary responses, where other inducible transcription factors are involved, it is not clear what these factors are and what DNA sequences are involved.

Many genes that are under the control of hormones have been used to study the mechanisms of hormone action in various systems. One of these genes is the rat α_{2u}-globulin gene, which was revealed to be under multi-hormone control in the 1970's.

α_{2u}-Globulin and its function.

α_{2u}-Globulin is a small 18.7 kd protein first described by Roy and Neuhaus (1966). This protein is abundant in the urine of adult male rats, but is absent from the urine of premature male or female rats at all ages. It contains a single polypeptide chain with 186 amino acid residues. A mature male rat excretes daily 25-30 mg of this protein through its urine (Roy 1979). In addition to its secretion in urine, α_{2u}-globulin is also present in tears and saliva of young rats of both sexes.

α_{2u}-Globulin is encoded by members of a multigene family. This multigene family consists of about 30 members, which are clustered on chromosome 5 (Kurtz 1981b; Dolan et al., 1982). Members of the family are very similar; each one consists of a 4 kb coding region containing 7 exons, 6 introns, and adjacent regulatory sequences. Transcription of the coding region produces a 1.3 kb mRNA. The sizes of individual λ genomic clones suggested that the spacer between members is at least 10 kb, but how the multigene family is organized is still under investigation.

The function of α_{2u}-globulin is not clear. Protein structure studies revealed that the α_{2u}-globulin protein contains a ligand-binding pocket which could bind small hydrophobic molecules. The shape of the pocket is consistent with the size and shape of a number of pheromones, indicating that this protein may function as a carrier of the pheromones which are involved in the animal's sexual attraction and aggression (Bocskei 1992). α_{2u}-Globulin is also involved in hyaline droplet nephropathy, an important toxicological syndrome in male rats resulting from exposure to certain industrial chemicals. These chemicals such as gasoline, d-limonene and 1,4-dichlorobenzene or their metabolites can bind α_{2u}-globulin to form a chemical-protein complex. The α_{2u}-globulin binds chemicals deep in the hydrophobic pocket (Borghoff 1991). The formation of a hydrogen bond between the chemical and specific amino acids deep in the pocket causes the α_{2u}-globulin to be less degradable, and the accumulation of α_{2u}-globulin in male rat kidneys leads to the development of kidney disease and cancer in rats that were exposed to these chemicals at a high level (Swenberg et al., 1989). The same exposure of female rats to these chemicals does not cause disease because female rats lack α_{2u}-globulin.

The major urinary protein (MUP) in mice is also encoded by a multigene family with about 40 members clustered on chromosome 4 (Bennett et al., 1982; Bishop et al., 1982). The hormonal and tissue specific regulation pattern of the expression of MUP gene in mice is similar to that of α_{2u}-globulin gene in rats in many aspects (Knopf et al., 1983). But there are some differences; for example, MUP is also synthesized in female mouse liver at 20% of the male level (Hastie et al., 1979). The MUP protein also has a ligand-binding pocket that is different in shape and deeper than that in α_{2u}-globulin. The shape and size of the ligand-binding pocket in MUP is still consistent with a number of pheromones, but it can not bind to the industrial chemicals. Therefore, at the same doses which cause kidney disease in rats, mice are unaffected. Since there are no homologues of α_{2u}-globulin in humans, chemicals that cause kidney disease via α_{2u}-globulin are not expected to cause the same problem in humans (Flamm et al., 1991, McClellan 1995).

The hormonal and tissue specific control of α_{2u}-globulin synthesis.

The major source of urinary α_{2u}-globulin is adult male liver (Roy and Neuhaus 1966). α_{2u}-Globulin represents 1-2 % of the total protein synthesis in the liver of adult male rats. The synthesis of α_{2u}-globulin in male liver is developmentally and hormonally controlled (Roy et al., 1983b). It is undetectable in the urine of male rats until the onset of puberty (about 31 days of age), and increases as the animal matures, reaching the adult level at about 60 days of age. This level of secretion is maintained throughout the male adult life, but decreases to nearly undetectable level in senescence (Roy et al., 1974). The synthesis of α_{2u}-globulin in male livers is induced by androgens, glucocorticoids, thyroid hormone, and growth hormone, and is strongly repressed by estrogens (Kurtz and Feigelson 1978). Its dependency on androgen is demonstrated by the study of castrated animals. Castration of male rats, which causes a deficiency of androgen, results in a sharp decrease in the synthesis of α_{2u}-globulin in the liver. Daily administration of androgen for 8 to 10 days can reinduce α_{2u}-globulin synthesis to control levels (Kurtz et al., 1976). Treatment of castrated (ovariectomized) female rats, which are deficient in estrogen, with either testosterone or 5α-dihydrotestosterone resulted in the induction of this protein (Roy and Neuhaus 1967). With a daily treatment of androgen for 2 weeks, a castrated female can secrete urinary α_{2u}-globulin from an undetectable level to about 15 mg/day, which is 2/3 of the secretion of a normal adult male. The androgen inducibility is age dependent. Androgen treatment of premature rats, either males or castrated females, does not induce α_{2u}-globulin (Roy 1973a).

Glucocorticoids can also induce the synthesis of α_{2u}-globulin. In contrast to the treatment of castrated male rats with androgen, which takes 8 to 10 days to reach the control level, administration of dexamethasone to castrated male rats results in a rapid induction of α_{2u}-globulin. The synthesis of α_{2u}-globulin becomes detectable within 3 hours, and reaches control levels in 24 hours. Induction of α_{2u}-globulin by

glucocorticoids is mediated by the hepatic glucocorticoid receptor. Administration of progesterone, which is known to compete with glucocorticoid for the glucocorticoid receptor, inhibits the induction of α_{2u}-globulin synthesis by glucocorticoid (Kurtz et al., 1978).

In addition to androgen and glucocorticoid. other hormones such as thyroid hormone, growth hormone, and insulin are also required for the synthesis of α_{2u}-globulin. Hypophysectomy of mature male rats, which is known to cause a multiple hormone deficiency, results in complete inhibition of α_{2u}-globulin synthesis. Treatment of hypophysectomized and castrated female rats with androgen did not induce α_{2u}-globulin. Complete recovery from the effect of hypophysectomy required treatment with a combination of four hormones: testosterone, corticosterone, thyroxine and growth hormone (Roy 1973b). Insulin also plays an important role in the synthesis of α_{2u}-globulin. Alloxan-induced diabetes in mature male rats results in an 80% reduction of α_{2u}-globulin synthesis. It can be reinduced to the control level by supplementation with insulin (Roy and Leonard 1973).

The effects of estrogen on the synthesis of α_{2u}-globulin are more complex than those of the other hormones. Initial exposure of castrated female rats to estrogenic hormones results in an increase in the hepatic concentration of α_{2u}-globulin which could be blocked with cycloheximide. On the first exposure, both 17β-estrodiol and 5α-dihydrotestosterone were almost equally effective in inducing α_{2u}-globulin. This induction is gradually lost after continued daily treatment (Roy 1977). On the other hand, daily treatment of mature male rats with estrogenic hormones brings about a large decrease in the hepatic synthesis of α_{2u}-globulin, a 50% decrease in 4 days and complete suppression of α_{2u}-globulin synthesis in 8 days. After complete suppression of α_{2u}-globulin synthesis with daily treatment of estrodiol-17β, withdrawal of estrogen treatment did not result in immediate reinitiation of α_{2u}-globulin synthesis. This estrogen-mediated quiescent period lasts over two weeks. Androgen supplementation of the estrogenized animals within this quiescent period could not induce α_{2u}-

globulin. Estrogen, when administrated with 5x concentration of 5α-dihydrotestosterone, was still be able to suppress completely the induction of α_{2u}-globulin synthesis (Roy et al., 1975). It has been reported that both androgen receptor and estrogen receptor are present in rat liver (Roy et al., 1974; Beers and Rosner 1977). Although estrogen can also bind to androgen receptor, it does not appear that the inhibition of α_{2u}-globulin synthesis by estrogen is due to its competition for androgen receptor. Administration of diethylstilbestrol, which is a synthetic estrogen and does not bind to androgen receptor, still inhibits the synthesis of α_{2u}-globulin (Roy 1979). It has been observed that the administration of estrogen causes a decrease of hepatic androgen receptor levels, but this decrease is slower than the decrease of α_{2u}-globulin and its mRNA (Kurtz and Feigelson 1978). This indicates that the estrogen receptor itself may play a role in the control of α_{2u}-globulin synthesis in the estrogen treated male rats.

α_{2u}-Globulin is also synthesized in salivary, preputial, and lachrymal glands of young rats. The synthesis of α_{2u}-globulin in these tissues exhibits a different pattern of regulation. In contrast to its delayed appearance in the liver, the synthesis of α_{2u}-globulin in salivary glands is detectable as early as 6 days of age in both males and females. The level of expression increases during development, reaching a maximum at day 30, followed by an abrupt decrease at puberty. The synthesis of α_{2u}-globulin in salivary glands is not affected by hypophysectomy, indicating that it is not controlled by hormones (Laperche et al., 1983; Gubits et al., 1984). Like those in the salivary gland, lachrymal α_{2u}-globulins were detected as early as day 10. The level of synthesis increases slightly with development, but there was no decrease in expression at puberty. Although α_{2u}-globulin can be found in lachrymal glands of both males and females, synthesis in the males is three- to five- fold higher than that in the females at all ages, and the synthesis in both males and females is affected by hypophysectomy (Gubits 1984; Held 1985). In the preputial gland, α_{2u}-globulin synthesis is also initiated at an early age. α_{2u}-Globulin constitutes the single most abundant protein, more than 20% of the total protein in this tissue. Unlike the strictly male-specific

hepatic synthesis of α_{2u}-globulin, expression of high levels of the α_{2u}-globulin mRNA in the preputial gland occurs in both sexes, and castration does not significantly alter the preputial concentration of this protein and its mRNA (Murty et al., 1987).

There is strong indication that the tissue specific, developmentally and hormonally regulated expression of α_{2u}-globulin is largely controlled at the transcriptional level. The level of transcription of α_{2u}-globulin genes in male rat liver is parallel with the level of α_{2u}-globulin synthesis during development. Estrogen treatment completely inhibits the transcription of α_{2u}-globulin. Hypophysectomy of male rats and hormone supplementation have also been proven to affect α_{2u}-globulin synthesis at the transcriptional level (Kurtz and Feigelson 1978; Kulkarni et al., 1985). The induction of α_{2u}-globulin by hormones is inhibited by cycloheximide, indicating that protein mediators, themselves possibly hormonally regulated, may play a role in this transcriptional control (Feigelson and Kurtz 1978; Chen and Feigelson 1979; MacInnes et al,1986; Addison and Kurtz 1986). Although the requirement of protein mediators suggests that part of the effects of steroid hormones on transcription may be indirect, direct interaction of steroid hormone receptors with specific binding domains in the 5'-upstream regions of α_{2u}-globulin genes has been demonstrated (Van Dijck et al., 1987; Van Dijck et al., 1989; Van Dijck et al., 1992). There are also indications that post-transcriptional regulation is involved in the control of α_{2u}-globulin gene expression. For example, glucocorticoids also regulate nucleo-cytoplasmic transport of RNA, in that reduced levels of glucocorticoid lead to the retention of α_{2u}-globulin mRNA in the nucleus and this retention is abolished after glucocorticoid injection (Fulton et al., 1985).

Although members of this gene family are highly similar, with 95% identity, different members of this gene family may contribute differently to the tissue-specific, developmentally, and hormonally controlled expression of this gene. Due to nucleotide differences, the proteins produced from different members are slightly different. These different isoforms of α_{2u}-globulin can be identified by

isoelectrofocusing. Multiple isoforms of α_{2u}-globulin have been detected in the liver and in the lachrymal, submaxillary, preputial, and mammary glands. Although many isoforms were common to more than one tissue, each tissue expressed a unique subset of isoforms. *In vitro* translation of mRNA from these tissues indicates that these isoforms of α_{2u}-globulin are the result of expression of different subsets of α_{2u}-globulin genes (MacInnes et al., 1986; Held 1985). The isoforms produced from liver mRNA did not increase to the same extent following hormone treatment. Some isoforms are more stringent than others in the androgen requirement, suggesting that even in the same tissue different members of the family may be regulated differently (Berry and Seelig 1986; Roy et al., 1987). The differential decrease of various isoforms during aging has also been noted (Roy et al., 1983).

Early studies of the mechanism of α_{2u}-globulin gene regulation.

The complexity of the regulation of α_{2u}-globulin synthesis provides an interesting system to study the processes of tissue specific, hormonal and developmental regulation of gene expression. But the large size of the gene family and the difficulty in identifying individual members make it difficult to unravel the mechanisms underlying the regulation. With the help of molecular technology, several members of the family have been cloned and studied in various systems. However our understanding of the regulatory mechanism is still very limited.

In vitro transcription of clone H1 revealed that the sequences within -74 bp of the start of transcription of this particular gene were sufficient for the basal level of transcription in the assay with liver extract from male and female rats. But there was no detectable transcription when spleen extracts from males or females were used (Sierra 1990). A binding site for nuclear factor C/EBP was located around -200 bp. But even with additional 5'-upstream sequences, up to -3000 bp, H1 failed to response with any sex specificity in this *in vitro* transcription system.

A 7 kb fragment of clone 207, which contains 2.7 kb 5'-upstream sequence, the coding region, and 300 bp downstream sequence, has been introduced into transgenic mice (Soares 1987). The transcription of clone 207 in the livers of the transgenic mice showed similar responses to hormones as those in the male rat liver. The transcription of this gene in male mouse liver was abolished by castration and fully restored after testosterone treatment. It was also induced in the livers of female mice by treatment with either testosterone or dexamethasone, following castration to eliminate the repressive action of estrogen. This indicates that the cis-acting regulatory elements responsible for the tissue-specific induction by testosterone or dexamethasone are closely linked to the gene (Soares 1987). *In vitro* transcription assays with liver extracts showed that, unlike clone H1, the fragment of -639 bp to +1395 bp from clone 207 can be efficiently transcribed only using male liver extracts and not with liver extracts from female or premature rats. The addition of liver extract from female rats does not inhibit the transcription of this fragment in male liver extracts (Sarkar and Feigelson 1989). This suggests that the sequences required for the male specific transcription are located within this region, and the absence of transcription in female extracts is not due to the presence of trans-acting inhibitor(s), but perhaps to the absence of trans-acting activator(s).

The analysis of the 5'-upstream region from clones RAP01 and RAO01 has shown that a fragment from these clones (-606 bp to -575 bp) is capable of binding estrogen receptor *in vitro* (Van Dijck and Verhoeven 1992). A fragment from clone RAP01 (-642 bp to -584 bp) has also been demonstrated to bind androgen receptor *in vitro*, although the same region from clone RAO01 showed no specific interaction with androgen receptor (Van Dijck et al., 1989). The same fragment from RAP01 also been shown to interact with glucocorticoid receptors and contains a 17 bp sequence homologous to the consensus sequence of a glucocorticoid binding site (Van Dijck et al., 1987). These data, like those of Soares, also suggest that the 5'upstream region of α_{2u}-globulin genes (up to -640 bp) plays an important role in their regulation by

hormones. In addition, they suggest that gene specific regulatory mechanisms may be operating.

Clone 91 was isolated from a rat genomic library in λ phage in 1981. When transfected into mouse L-cells, clone 91 is inducible by dexamethasone (Kurtz 1981). A fragment of this clone, which contained 235 bp 5'-upstream sequence, the coding region, and 400 bp of 3'-downstream sequence, was reported to be induced by dexamethasone in L-cells (Kurtz et al., 1982). It appears that the induction of clone 91 by glucocorticoid in this L-cell system is a secondary response since protein synthesis was required before the hormone could exert its stimulatory effect on α_{2u}-globulin transcription. Linker-scanning mutagenesis of region -235 bp to -20 bp revealed that the sequences between -115 bp and -160 bp are required for the induction by dexamethasone (Addison and Kurtz 1986). These sequences were found to be protected by a nuclear factor present in liver nuclei of both male and female rats (Addison and Kurtz 1989). But the footprint produced was not male liver specific, and was also produced with extracts of L-cells, male kidney and female liver. The analysis of footprints within region -220 to -110 did not reveal any differences between male liver extracts and female liver extracts. It was also unable to reveal any differences with the addition of dexamethasone (Addison and Kurtz 1989), indicating that sequences that interact with glucocorticoid receptor or glucocorticoid induced factors may be located outside this 250 bp region.

This thesis continues the work on the regulation of clone 91 in mouse L-cells. It consists of three chapters. The first chapter details the induction of these clones by dexamethasone. In addition to the -235 bp upstream sequences reported previously that are required for dexamethasone induction, the -700 to -235 bp region has been found necessary for maintaining a high level of induction by dexamethasone. Sequence analysis has revealed a glucocorticoid receptor binding site in this region. Clone #2, which contains a base substitution at this binding site showed very little response to dexamethasone. In the second chapter, the response of clone 91 to estrogen in the

mouse L-cell system, and the sequences required for the response will be presented. The effect of estrogen on the induction by dexamethasone will also be discussed here. Chapter 3 describes the attempt to identify the tissues in which clones 91 and #2 may be expressed *in vivo*. It includes the analysis of α_{2u}-globulin mRNA from different tissues of rats by RT-PCR and a characterization of the RT-PCR products with three restriction enzymes to reveal the tissue specificity of α_{2u}-globulin transcripts. A future perspective of the research of α_{2u}-globulin gene will be presented at the end.

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Chapter 1

An analysis of the 5'-upstream sequences required to maintain a high level induction of the α_{2u}-globulin gene, clone 91, by dexamethasone.

Introduction

Gene expression regulated by glucocorticoids has been studied in many systems (Ringold 1983; Rousseau 1984). In the regulation processes, hormone-activated receptors bind specific DNA sequences, the so called glucocorticoid response elements (GRE), which are typically present in the promoters of hormone-responsive genes (Payvar et al., 1983; Godowski et al., 1989; Evans 1988; Yamamoto 1985; Beato et al., 1989). The consensus sequence for the GRE is 5'-AGAACANNNTGTACC-3' (Truss and Beato 1993), where the hexanucleotide sequence AGAACA is essential for binding of the hormone-receptor complex to DNA (Tsai et al., 1988; Mangelsdorf et al., 1995). Upon binding to the GRE, the receptor interacts with the transcription complex to either stimulate or suppress the transcription activity. One of the well demonstrated examples of glucocorticoid-regulated transcription is mouse mammary tumor virus (MTV). Fragments of MTV DNA can bind selectively to the glucocorticoid receptor *in vitro* (Scheidereit et al., 1983; Payvar et al., 1983). The GREs present in these fragments act as receptor-dependent transcriptional enhancer elements *in vivo* (Chandler et al., 1983; Ponta et al., 1985).

The expression of the α_{2u}-globulin gene in male adult rats is under multi-hormonal control. The effect of hormones such as androgen, glucocorticoid, and growth hormone on the expression of this gene *in vivo* has been well documented (reviewed by Kurtz and Feigelson 1978; Roy 1979). Castration of male rats, which causes a deficiency of the sex hormones, results in deceased expression of this gene. Treatment of castrated male rats with dexamethasone results in a rapid induction of α_{2u}-globulin synthesis. The level of expression falls back to the control level in 24 hours. This induction of α_{2u}-globulin by glucocorticoids is mainly at the transcription level, and it is mediated by the hepatic glucocorticoid receptor. Administration of progesterone, which is known to compete with glucocorticoid for the glucocorticoid receptor, inhibits the induction of α_{2u}-globulin synthesis by glucocorticoid (Kurtz et al., 1978).

The mechanism underlying the glucocorticoid induction is not clear. Several studies have been conducted to determine the sequences and transcription factors involved in the induction. A region from clone RAP 01, between -642 and -452 bp upstream of the transcription start site, showed significant binding affinity to the glucocorticoid receptor *in vitro*. However the same region from clone RAO 01, which has a number of base substitutions compared with RAP01, showed no significant binding (Van Dijck et al., 1987). A fragment from clone 207, from -639 bp to +1395 bp, has been introduced into transgenic mice. The transcription of this fragment in the liver of the transgenic mice showed hormonal responses similar to those in the male rat liver (Soares 1987). The expression of a hybrid coding region driven by this promoter region can be efficiently induced by dexamethasone in castrated mice (Sarkar and Feigelson 1989). These data suggest that the sequences up to -650 bp may play an important role in the glucocorticoid induction.

Clone 91 has been shown to be inducible by dexamethasone in an *in vivo* system, mouse L-cells (Kurtz 1981). The 5'-upstream sequence up to -235 bp of this clone, when linked to a HSV TK reporter gene, was reported responsive to dexamethasone treatment (Kurtz et al., 1982). Studies with plasmid 91R91, which was reported to contain 500 bp

5'-upstream sequences (it is actually 762 bp as discussed later in this chapter), the coding region and 400 bp 3'-downstream sequences of clone 91, showed that the induction of this gene by glucocorticoid in the L-cell system is inhibited by cycloheximide (Addison and Kurtz 1986). Linker-scanning mutagenesis of region between -235 bp and -20 bp, which was conducted with plasmid 91R91, revealed that the sequences between -115 bp and -160 bp are necessary for the induction by dexamethasone (Addison and Kurtz 1986). Several nuclear factor binding sites have been located in this region by footprints (Addison and Kurtz 1989), but none of these footprints was specific to male liver extracts or extracts from L-cells treated with dexamethasone. They were also produced with extracts from female rat liver or L-cells without dexamethasone treatment, indicating that the binding sites in this -235 bp to -20 bp region are the binding sites for basal transcription factors. The sequences responsible for dexamethasone induction of clone 91 are still not properly defined and this chapter presents the results of further investigation that we have undertaken.

Materials and Methods

Materials: Tissue culture medium, calf serum, geneticin, and Taq DNA polymerase were purchased from GIBCO-BRL. Restriction enzymes were purchased from New England Biolabs and GIBCO-BRL. S1 nuclease and a T7 Sequencing Kit were purchased from Pharmacia Biotech. Guanidinium thiocyanate and dexamethasone were purchased from Sigma Chemical Co. γ-^{32}P-ATP and α-^{32}P-dATP were supplied by Amersham Corp. Zeta-probe nylon membrane was purchased from Bio-Rad Laboratories.

DNA Manipulation: Bacteria and λ phage culturing, plasmid DNA and λ phage DNA preparation and subcloning were performed according to Sambrook et al.,(1989).

Genomic Library Screening: A commercial rat genomic library in λ DASH II was screened with an end labeled oligonucleotide KF40, which covers -8 bp to +32 bp

encompassing the transcription start site of 91R91 (Fig 1-1), according to the procedure provided by Stratagene. Positive plaques were isolated and subjected to a further PCR test. The oligonucleotides used in the PCR test were: KWO1 (5'-CTTTATACTCTCTCTGGGTTT-3'), KWO2 (5'-CCTTCTCATATGGGCCTCCA-3'), and KWO3 (5'-AGATGACATCGCCAAGTTTC-3'). Single phage plaques were isolated and suspended in 12 µl of H_2O. Four µl of this suspension was mixed with 3 µl 10x PCR buffer, 4 µl of 1 mM dNTP, 2 µl of 1 µM KWO1, 2 µl of 1 µM KWO3, 7 µl 30 mM magnesium chloride, 0.5 µl Taq DNA polymerase, and 7.5 µl H_2O. PCR was performed under the following conditions: 1 cycle of 5 min at 95°C, 1 min at 58°C and 2 min at 72°C, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C and 2 min at 72°C.

DNA sequencing: DNA fragments were subcloned into pBluescript KS^+ and sequenced with the T7 Sequencing Kit from Pharmacia Biotech following the manufacturer's instructions. A typical sequencing strategy is illustrated in Figure 1-2. The primers used in sequencing were: T3 promoter primer, T7 promoter primer, KWO1, KWO2, and KWO3.

Plasmid construction: Plasmid 91R91 contains a portion of the 5'-upstream region, all of the coding region and 400 bp 3'-downstream region of the original clone 91 α_{2u}-globulin gene inserted into pBR322 (Fig 1-3). The HindIII site in this plasmid (marked with * in Fig. 1-3), which connects pBR322-derived vector sequences and the cloned α_{2u}-globulin gene sequences, was replaced with a XhoI site by partial digesting 91R91 with HindIII, refilling the sticky ends and ligating in a XhoI linker. The consequent plasmid was named 91RX, and the vector part in this plasmid was named RX. This vector was then used to construct all the plasmids used for transfection purposes. Plasmids 91RX2, 91RX3, and 91RX4 (Fig. 1-4) were constructed by replacing the 5'-upstream fragment from XhoI to XbaI in 91RX, which is identical to the HindIII to XbaI fragment in 91R91, with different 5'-upstream fragments from pL21. The isolation of pL21 which contains an extended 5'-upstream region of clone 91 will be discussed later. Plasmid 91Da (Fig 1-4) was constructed by subcloning clone 91 sequences containing 226 bp 5'-upstream

sequence of clone 91 and the same coding region and 400 bp 3'-downstream region into vector RX.

Transfection of mouse L cells. Mouse L tk^- $aprt^-$ cells (Weglier et al., 1979) were transfected by a modified method of Ausubel et al., (1995). One day before transfection, confluent cells from one 100 mm plate were split 1: 10 in Dulbecco modified minimum essential medium (DMEM) plus 10% calf serum. Cells were fed with 9 ml of the same medium two hours before transfection. Cells in each plate were treated with 1 ml of calcium phosphate precipitate containing 15 µg of plasmid DNA, 1 µg of the neomycin resistant plasmid pKOneo (Dr. Hanahan, Cold Spring Harbor Laboratory), and 15 µg of L-cell carrier DNA. After 16 hours of incubation, cells were washed twice with PBS and fed with DMEM plus 10% calf serum. Cells were incubated for 48 hours then fed with selective medium which contains 400 µg/ml of Geneticin. After two weeks of selection, single colonies were isolated, and further incubated as cell lines. Each cell line was examined for the presence of α_{2u}-globulin DNA by Southern blotting, and the transcription of α_{2u}-globulin RNA was examined by S1 protection.

Genomic DNA preparation and Southern blotting. Tissue culture cell genomic DNA was extracted from one plate of cells according to Ausubel et al., (1995). Twenty µg of DNA from each cell line was cut with an appropriate restriction enzyme, fractionated on a 0.8% agarose gel and transferred to a Zeta-probe nylon membrane according to the manufacturer's instruction. The probes used in Southern blotting was the 4.2 kb HindIII α_{2u}-globulin gene fragment from plasmid 91R91 (see Fig. 1-3) labeled with α-^{32}P-dATP.

Induction of α_{2u}-globulin RNA. Cells containing the α_{2u}-globulin gene were fed with DMEM + 10% calf serum and split 1:8 the day before induction. Two plates of cells were used in each sample group. In the control group, cells were fed with DMEM+ 10% calf serum. In the experimental groups, cells were fed with DMEM + 10% calf serum + 0.05 M dexamethasone. The respective media were replaced every two days and the cells were harvested on the ninth day of induction for RNA isolation.

In the dexamethasone withdrawal experiment, cells in the control group were fed with dexamethasone on the 0, 2nd, 4th, 6th, 8th, and 10th day, and two plates of cells were harvested on each of the following days: 1st, 3rd, 5th, 7th, 9th, and 11th. For the Dex(-4) experimental group, a total of eight plates of cells were washed three times with medium on the 4th day after induction and fed with medium without dexamethasone on the same feeding schedule as those in control group. Two plates of cells were harvested on the 5th, 7th, 9th, and 11th days. For the Dex(-6) group, dexamethasone was withdrawn on the 6th day of induction following the same treatment as Dex(-4) group, and cells were harvested on the 7th, 9th, and 11th days. Dexamethasone was withdrawn from Dex(-8) group on the 8th day of induction, and cells were harvested on the 9th and 11th days. All collected cells were carried through the homogenization step for RNA isolation. Samples that were homogenized were either frozen at -70°C or processed through the subsequent steps of RNA isolation.

Total RNA isolation. Cells from two 100 mm tissue culture plates were homogenized in 7 ml guanidine solution which contains 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7), 0.1 M 2-mercaptolethanol, 0.5% sarkosyl (Ausubel et al., 1995). After extraction with phenol/chloroform (1:1), samples were loaded into SW41Ti ultracentrifuge tubes (Beckman, polyallomer), which contained 3 ml of 5.7 M cesium chloride, and centrifuged at 29,000 rpm for at least 16 hours. The RNA pellets were suspended in 400 µl buffer containing 5 mM EDTA, 0.5% sarkosyl, and 5% 2-mercaptoethanol. After extracting once with 1:1 phenol/ chloroform and once with chloroform, the RNA was precipitated with 2.5 volumes of ethanol and the pellets were then resuspended in 600 µl DEPC treated H_2O.

S1 protection assay. Total RNA (25 µg) from each sample was used in a quantitative S1 protection assay (Addison and Kurtz 1986). The probe used in the S1 protection assay was the oligonucleotide KF40 mentioned above, labeled with γ-^{32}P-ATP using T4 polynucleotide kinase. Total RNA (25 µg) was hybridized with 0.02 pmole of the

radiolabeled KF40 oligonucleotide in 20 µl hybridisation buffer containing 10 mM Tris-HCl (pH7.4), 1 mM EDTA, 0.4 M NaCl, and 0.1% SDS, by heating at 90°C for 2 min., followed by a 1 hour incubation at 65°C. S1 digestion solution (250µl) containing 0.6 M NaCl, 30 mM sodium acetate (pH 4.8), 1 mM $ZnSO_4$, 0.5% glycerol, 20 µg/ml salmon sperm DNA(denatured), and 100 unit of S1 nuclease was then added. After a 1 hour incubation at room temperature, 75 µl of stop solution containing 2 M of ammonium acetate, 50 mM of EDTA and100 µg/ml of salmon sperm DNA (denatured) was added. Samples were precipitated with 1 ml 95% ethanol. Protected probe was fractionated on a 15% acrylamide/urea denaturing gel and located on the gel using X-ray film (Kodak or Fuji). The 32 base protected fragment was cut out and quantitated in a scintillation counter using a Cherenkov mode.

Results

Cloning of 5'-upstream sequences of clone 91. Plasmid 91R91 (Fig. 1-3), which is derived from clone 91, was used in the studies described in Addison and Kurtz (1986). The 5'-upstream sequence of the α_{2u}-globulin gene in this plasmid was believed to be 500 bp, but was found to be 762 bp following the sequencing discussed later in this chapter. The sequence up to -235 bp was determined and this region was analyzed by linker scanning mutagenesis (Addison and Kurtz 1986). This region was chosen since it had been previously reported to be sufficient for the induction of this gene by dexamethasone (Kurtz et al., 1982). In order to examine the influence of 5'-upstream sequences of clone 91 beyond -235 bp on the induction of α_{2u}-globulin by glucocorticoids, a rat genomic library in λ DASH II was screened for clones with sequences that are identical to these in 91R91. The library was screened primarily with KF40. Positive clones were isolated and screened by PCR with 91R91 specific primers: KWO1, and KWO3 (Fig. 1-1), to detect the presence of sequences that are specific for the clone 91 gene. These two oligonucleotides are located between -230 bp and +60 bp, a highly divergent region of the 13 members of the α_{2u}-globulin gene family characterized

(Addison, unpublished). Five λ clones produced bands with the predicted size in the PCR reaction (Fig. 1-5). The genomic sequences from these five positive clones were isolated and subcloned into pBluescript. The consequent plasmids were named pL followed by the same number as the λ clones. These five clones, pL21, pL2, pL8, pL9 and pL10 were then subjected to extensive restriction mapping (Fig. 1-6). Four of these five clones, clone pL2, pL8, pL9 and pL10, have the full length coding region of α_{2u}-globulin gene, with various lengths of 5-upstream sequences and 3'-downstream sequences. Their restriction maps are similar, especially in the regions that are close to the coding region. But they all lack the EcoRI site located in the second exon of 91R91 (Fig. 1-3). Clone pL21 has only a partial coding region, but it has the same restriction sites for Hind III, Xba I and EcoR I as those in 91R91.

Sequence analysis of pL21 showed that from –238 bp to +63 bp, its sequence is identical to the sequence in 91R91 (Fig.1-7). Since sequences in this region are highly variable among members of the α_{2u}-globulin gene family, compared with other regions, we concluded that the inserts in pL21 and 91R91 are from the same member of the gene family. The sequences from plasmids 91R91 and pL21 were then combined to construct plasmids with varying lengths of the 5'-upstream region and a full length coding region of clone 91 for further study.

Full induction of clone 91 by dexamethasone requires 5' sequences upstream of -235 bp. Three plasmids containing 2.7 to 6.3 kb 5'-upstream sequences of the clone 91 were constructed (Fig.1-4). These plasmids, together with plasmids 91R91 and 91Da, were used to transfect mouse L-cells as described in Materials and Methods. Cloned cell lines from transfectants of each construct were isolated and tested for the presence of the α_{2u}-globulin gene by Southern blotting as illustrated in Figure 1-8. The effects of dexamethasone on α_{2u}-globulin expression in several of the cell lines that had tested positive were then examined by the S1 protection assay.

In the S1 protection assay, α_{2u}-globulin RNA was quantitated by the radioactivity present in the 32 base fragment, which is the protected part of KF40, the oligonucleotide used for this analysis (see Fig 1-1). A typical result is shown in Figure 1-9. The 40 base fragment that appears on the autoradiograph is not due to insufficient digestion with S1 nuclease, since there is no sign of the ladder produced by insufficient digestion. It results from the protection by an unknown RNA crossing the transcription initiation site of α_{2u}-globulin gene. This unknown RNA is present in the RNA of the α_{2u}-globulin-bearing transfected cell lines, as well in the RNA of male rat liver, but not that of female liver. Only very low amounts are present in the RNA of mouse L-cells (Fig. 1-9). It may be related to the transcript detected earlier that is 77 bp longer than the principle transcript that produces the 32 base fragment (Winderickx et al., 1987). Unlike the 32 base fragment, the amount of this unknown RNA is neither affected by hormone treatment nor affected by 5'-upstream sequence deletions. The presence of this fragment in transfected L-cells is a good indicator that the introduced α_{2u}-globulin gene is integrated into a transcriptionally active region and not a heterochromatic region of the genome.

The inducibility of α_{2u} globulin gene by dexamethasone in cell lines transfected with the different constructs shown in Figure 1-4 are summarized in Table 1-1. Due to varying position effects at the different insertion sites, the level of induction differs among cell lines transfected with the same plasmid. The level of induction in the expressing cell lines does not correlate directly to the copy number of α_{2u}-globulin genes integrated into the genome. Some of the cell lines transfected with plasmids 91RX2, 91RX3 and 91RX4 do not express either of the transcripts of the introduced gene. But on average, the α_{2u}-globulin genes in cell lines transfected with constructs containing at least 762 bp 5'-upstream sequences are highly inducible by dexamethasone (Fig.1-10). The average fold-induction among these cell lines is between 16 and 24. In contrast, cell lines transfected with 91Da, which contains 226 bp of 5'-upstream sequence exhibit a dramatic reduction of α_{2u}-globulin RNA induction to about 4 fold. Since the α_{2u}-globulin gene in these cell lines was integrated into a transcriptionally active region, as indicated by the

strong signal of the unknown RNA, it can be concluded that the loss of 5'-upstream sequences between -226 bp and -762 bp was responsible for this dramatic decrease.

The 4-fold increase of α_{2u}-globulin mRNA by dexamethasone treatment in cell lines transfected with 91Da is similar to the 2 to 4-fold increase that Kurtz observed with a reporter gene driven by the 236 bp 5'-upstream sequence of clone 91 (Kurtz et al., 1982). Since footprinting studies of this region failed to identify any dexamethasone induced binding sites, this increase of α_{2u}-globulin mRNA is likely due to the stabilizing of the mRNA by dexamethasone which has been observed in other transcription systems (Petersen 1989; Murasawa et al., 1995).

Sequencing of the -762 bp to -220 bp 5'-upstream region did reveal that at two sites in this region some homology to the consensus sequence of the glucocorticoid receptor binding site was present (Fig. 1-11). The one at -640 bp contains two half sites that are similar to the half site of glucocorticoid response element (GRE), separated by a two base pair spacer, instead of the usual 3 base pairs. The second one at -513 bp contains a perfect AGAACA half site at one end, which is presumed to be essential for glucocorticoid receptor binding to DNA (Tsai et al., 1988). The sequence of clone 91 between -642 bp and -452 bp is identical to the same region in clone RAP01 which has been demonstrated capable of binding glucocorticoid receptor *in vitro* (Van Dijck et al., 1987), suggesting that the sequence in this region of clone 91 is also capable of binding glucocorticoid receptor. It can be inferred that the decreased response of α_{2u}-globulin genes to dexamethasone in cell lines transfected with 91Da, compared to the response in 91R91 transfected cells, is due to the loss of glucocorticoid receptor binding sites in this region.

The inducibility of α_{2u}-globulin RNA in four other clones, pL2, pL8, pL9 and pL10 (Fig.1-6), by dexamethasone was also tested. Surprisingly all of them showed less than 3 fold responses to dexamethasone treatment when constructs containing 2.7 kb or 764 bp 5'-upstream sequences were used (Fig. 1-12). Sequencing of the 5'-upstream

regions of these clones showed that from -1047 bp to +50 bp, or to -119 bp in the case of pL8, they have the identical sequences (Fig. 1-13). So it is likely that they come from the same member of the α_{2u}-globulin gene family, that I have named clone #2. From -719 bp to +52, the sequence in clone #2 is slightly different from that in clone 91 (Fig. 1-14), with 45 bases differing in this region. One of the base substitutions is at the AGAACA half site of the second glucocorticoid response element at -513 bp. The sequence at the other possible binding site (-640 bp) is still identical to that in clone 91. A sequence comparison (see Fig.1-14) revealed that the sequences around the two possible glucocorticoid receptor binding sites in clone #2 are identical to that in clone RAO01, although differences elsewhere prove these clones represent different members of the gene family. RAO01 did not bind to glucocorticoid receptor *in vitro* (Van Dijck et al., 1987). Thus the loss of inducibility of clone #2 is correlated with both the altered GRE and the loss of receptor binding *in vitro*. This strongly suggests that the 5'-distal GRE is not an active binding site for the receptor, whereas the site at -513 bp is active.

In an attempt to test whether a direct binding of the hormone-receptor to the α_{2u}-globulin DNA is required to effect an increase in the mRNA, a dexamethasone withdrawal experiment was conducted. Since the active form of the receptor is the hormone-receptor complex (Muller and Renkawitz 1991), the withdrawal of dexamethasone is expected to inactivate the glucocorticoid receptor. If direct binding of the glucocorticoid receptor to the α_{2u}-globulin gene is required to induce transcription, the withdrawal of dexamethasone would trigger a rapid decrease in transcription. On the other hand, since dexamethasone induction of the α_{2u}-globulin gene in clone 91 has been demonstrated as a secondary response (Addison and Kurtz 1986), its transcription clearly requires the synthesis of other transcription factors. If α_{2u}-globulin transcription does not also require direct binding of the glucocorticoid receptor to the DNA, the withdrawal of dexamethasone would produce a slow decrease of transcription activity due to the gradual loss of the other transcription factors. The experiment showed that the withdrawal of dexamethasone from the culture medium indeed triggered a dramatic decrease of transcription activity (Fig. 1-15). This indicates that binding of the glucocorticoid

receptor to DNA sequence in clone 91 is required for the hormonally induced transcription.

Discussion

In general, glucocorticoids regulate the expression of a given gene through regulating the transcriptional rate. The hormone-activated receptors bind to the GRE(s) in the promoter region, and interact either directly (Tsai and O'Malley 1994) or via other bridging factor(s) (Cavailles et al., 1995: Halachmi et al., 1994) with the transcription initiation complex to either stimulate or suppress the transcription of the target gene.

In the studies reported here, it is shown that the -762 bp 5' upstream sequence of α_{2u}-globulin gene in clone 91 is sufficient for the high level of induction of this gene by glucocorticoid in mouse L-cells. Addition of 5'-upstream sequences beyond -762 bp did not alter the level of induction significantly. However, deletion of the DNA between -762 bp and -226 bp of this gene dramatically reduced the inducibility of the α_{2u}-globulin gene (Table 1-1, Fig 1-10). Several lines of evidence suggest that the glucocorticoid GRE at -513 bp is likely responsible for the hormone inducibility. First, clone #2 which is non-responsive to dexamethasone contains a GRE at -513 bp which differs from that found in the inducible clone 91 (Fig 1-13). Secondly, the clone #2 version of this GRE is identical to that in RAO01, which did not bind to glucocorticoid receptor *in vitro*. Conversely, the clone 91 version of the GRE is identical to that in RAP01 (Fig. 1-14), which did bind to the glucocorticoid receptor (Van Dijck et al., 1987).

The induction of α_{2u}-globulin RNA in clone 91 by glucocorticoid requires the constant presence of the hormone-activated receptor as showed in the dexamethasone withdrawal experiment (Fig. 1-15). From this result, we can conclude that the hormone-receptor complex binds to the -513 bp GRE as a necessary but not sufficient requirement for activation of the α_{2u}-globulin gene. Since the induction also requires protein synthesis

as demonstrated in earlier studies (Addison and Kurtz 1986; Chen et al., 1979), the binding of the glucocorticoid receptor to the promoter region and the presence of other coactivators are required for α_{2u}-globulin expression.

The S1 protection assay used in examining the expression of α_{2u}-globulin gene quantitates the accumulation of the α_{2u}-globulin mRNA present in the total RNA and is therefore not a direct measure of the rate of transcription of the gene. Nuclear run-off transcription assay which do measure the rate of transcription (Bentley and Groudine 1986) were not carried out in this thesis. However, the base substitution in clone #2, which is located in the GRE at -513, would not have affected the stability of the mRNA and yet resulted in a loss of induction. This suggests that the mRNA accumulation measured by S1 protection reflected the promoter activity of the α_{2u}-globulin gene.

The data presented in this chapter still leave unanswered a number of questions about the mechanism of induction. The 3 day lag period of the glucocorticoid induction of α_{2u}-globulin gene in clone 91 (Addison and Kurtz 1986) suggested that coactivators induced by glucocorticoid are involved in the induction. So far none of these factors has been cloned or purified in the α_{2u}-globulin gene system or any other glucocorticoid induced transcription system for that matter. It is not clear whether or not these coactivators are DNA binding proteins, and whether or not they would interact with glucocorticoid receptor complex. Although the 5'-upstream sequence, between -762 bp and -226 bp, of the α_{2u}-globulin gene in clone 91 has been proven to be important for glucocorticoid induction, and glucocorticoid receptor binding to this region can be inferred, it may also contain the binding sites for coactivators. The footprints in the -230 bp to -20 bp (Addison and Kurtz 1986) did not reveal any binding sites for glucocorticoid inducible factors and probably comprise factors involved in the basal transcription complex.

The altered glucocorticoid receptor binding site at -513 bp in clone #2 may be responsible for the loss of inducibility of clone #2 by dexamethasone. But it is still

possible that other base substitutions also contribute to this loss. Four of the 45 base substitutions that destinguish clone 91 and #2 are located at a nuclear factor binding site in -115 bp to -160 bp region (Addison and Kurtz 1989). Although Addison has shown this region is important for glucocorticoid induced transcription, the binding of this factor is not sensitive to hormone treatment, implying that this factor is part of the basal transcription complex. How these base substitutions would affect the binding of this nuclear factor is unknown. A more detailed analysis of this 5'-upstream region, especially the base substitutions in the 5'-upstream regulatory region between clone 91 and clone #2 should be carried out to characterize the nature of the factors involved and the mechanism of glucocorticoid induced transcription.

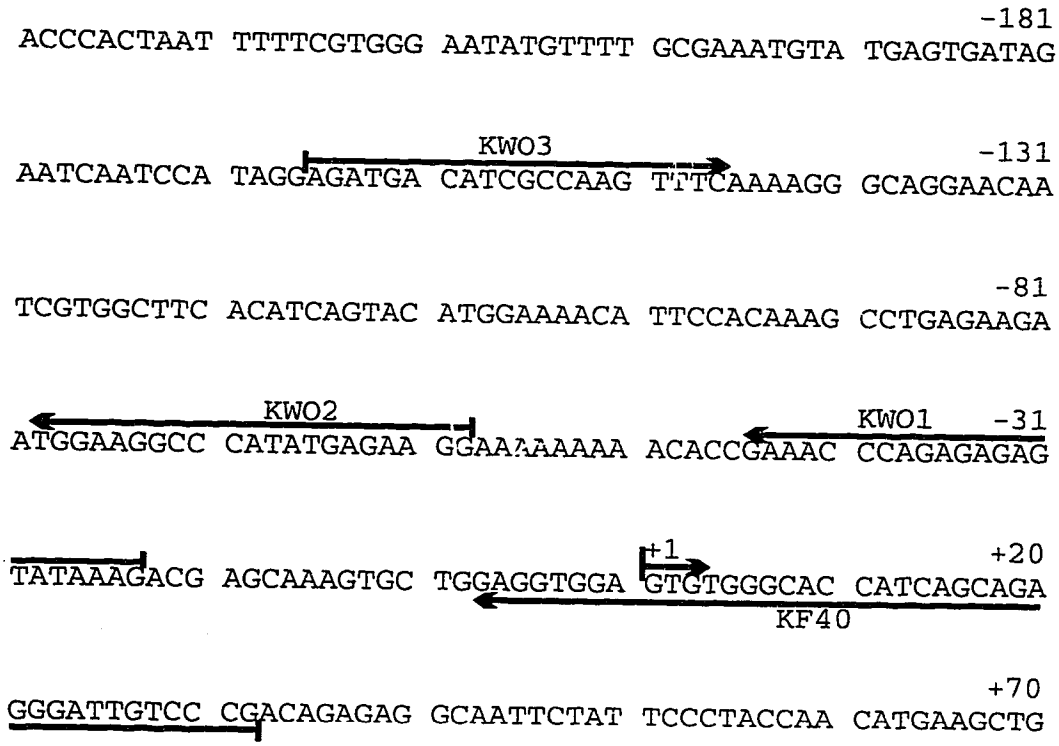


Figure 1-1. 5'-upstream sequences of clone 91 and related oligonucleotides. Sequences are numbered according to the transcription initiation site, +1. Oligonucleotides and their polarities are indicated by arrows. The 40 nucleotide KF40 was used in λ library screening and for the S1 protection assay. KWO1 and KWO3 were used in PCR screening for clone 91 sequences, and DNA sequencing. KWO2 was also used in DNA sequencing.

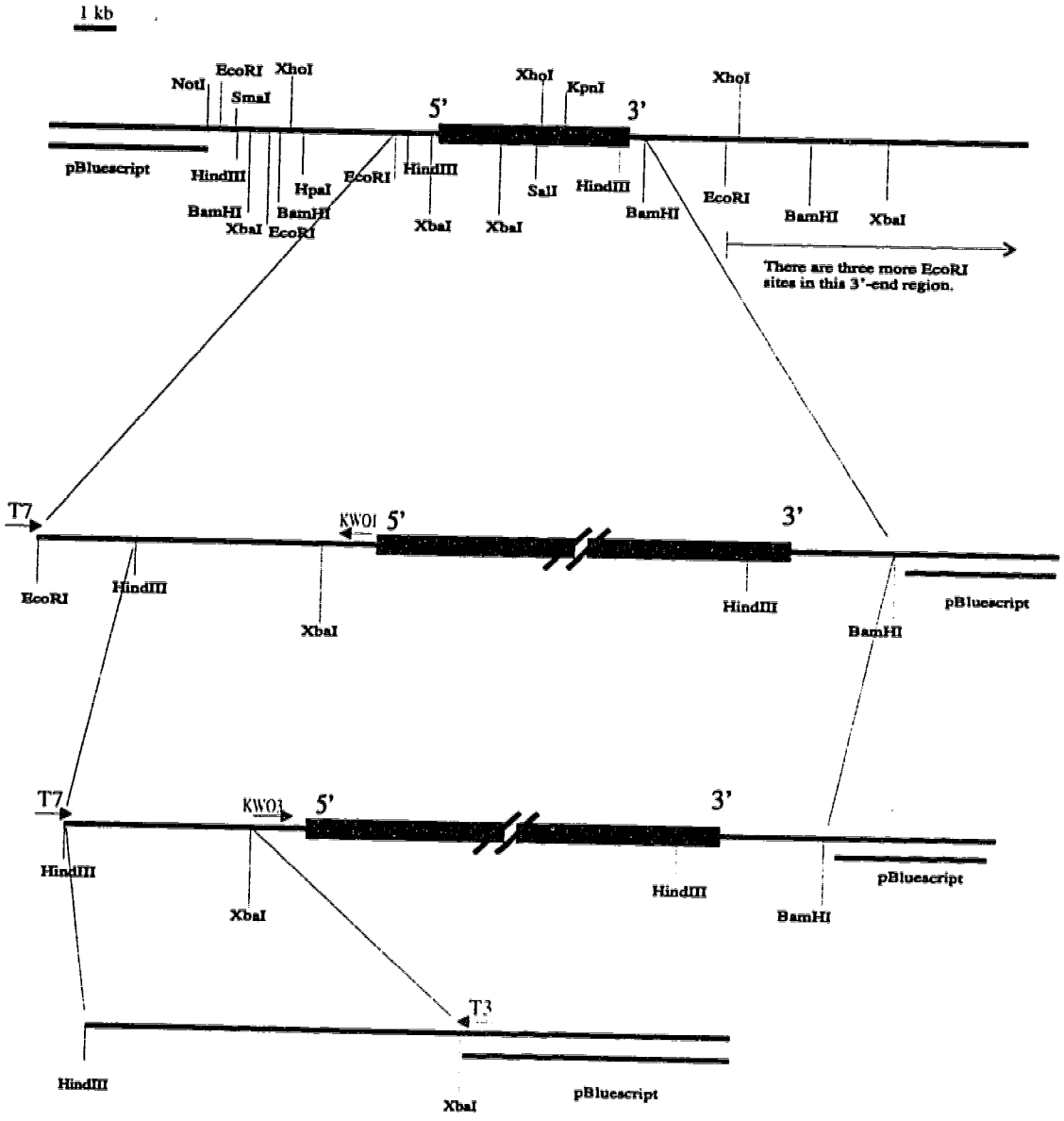


Figure 1-2. A typical strategy for DNA sequencing. Several subclones were made from the original clone, here for example pL2, with vector pBluescript. Sequencing was performed by using either T7 promoter primer (T7) or T3 promoter primer (T3) from the vector sequence, or using internal primers KWO1 and KWO3.

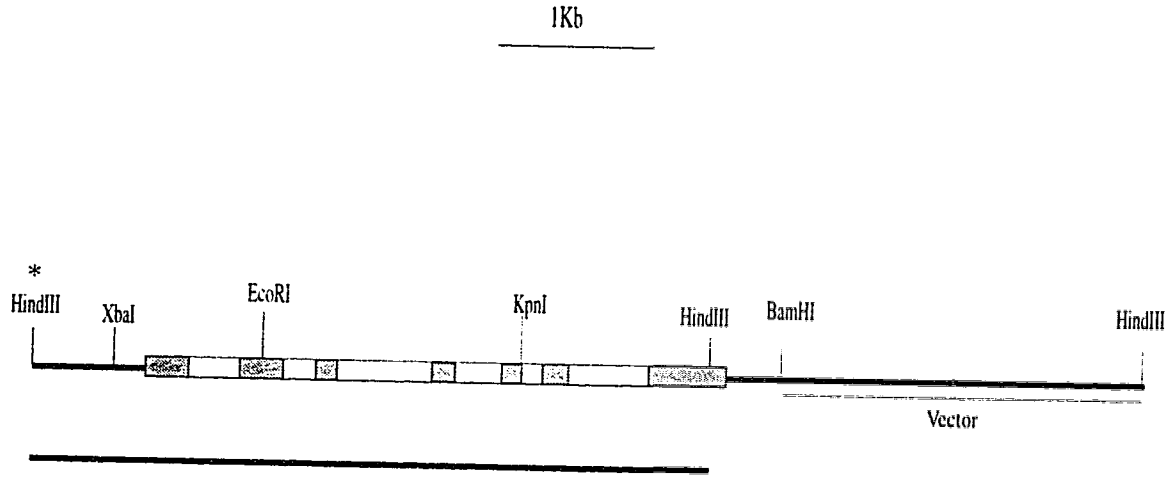
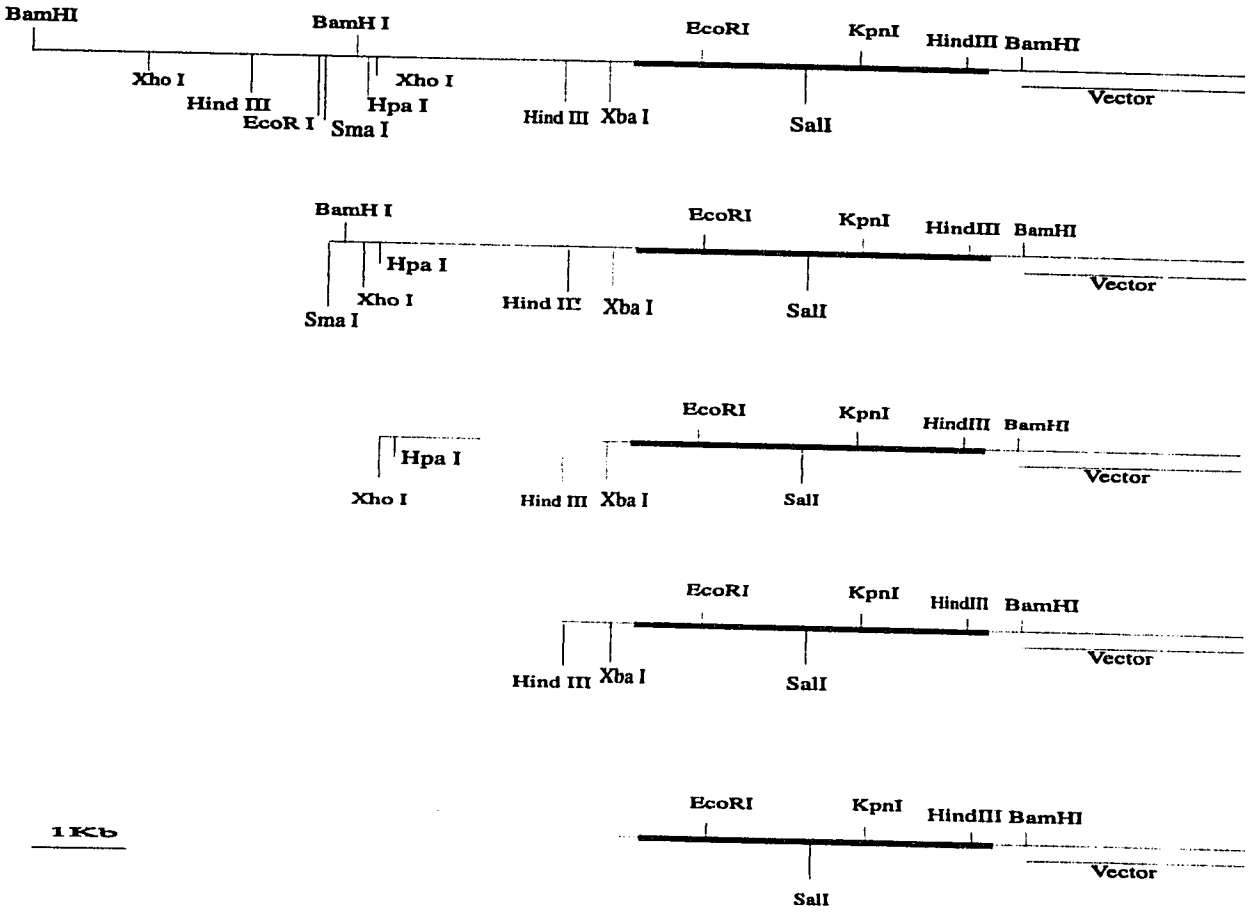


Figure 1-3. Restriction map of 91R91, redrawn from Kurtz et al.,(1981). The open bars are introns and the closed bars are exons of the α_{2u}-globulin gene, clone 91. The vector sequence is a derivative of pBR322. The (*) indicates the HindIII site removed to facilitate the substitution of a nested set of 5'-upstream regions.



	5'-upstream sequences
91RX4	6.3 kb
91RX2	3.2 kb
91RX3	2.7 kb
91R91	762 bp
91Da	226 bp

Figure 1-4. Restriction maps of constructs containing the coding region of clone 91 and different lengths of 5'-upstream sequences. The vector used for all constructs was vector RX, derived from pBR322. The bold line indicates the coding region of the α_{2u}-globulin gene. The 3'-downstream sequences are about 400 bp. The length of 5'-upstream sequences in each construct is indicated on the right side.

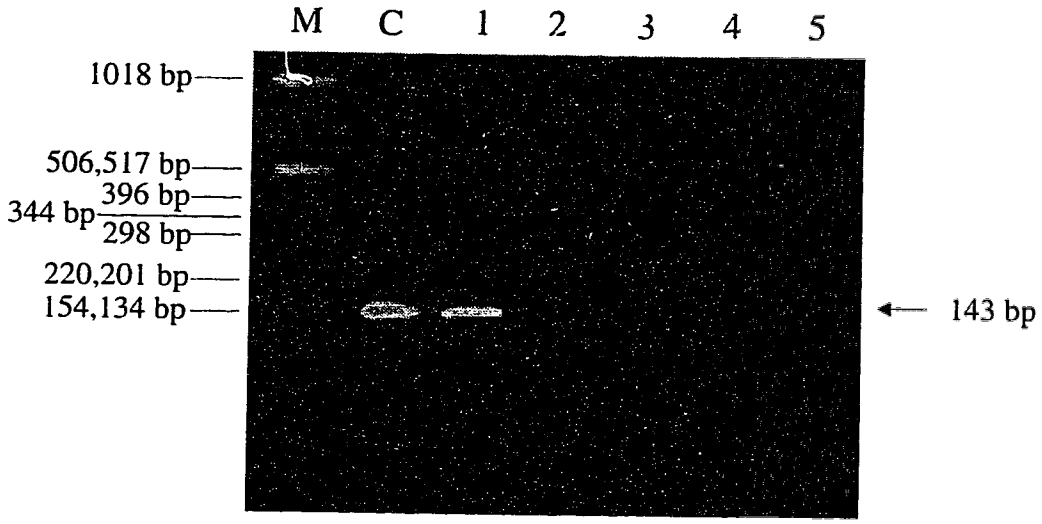
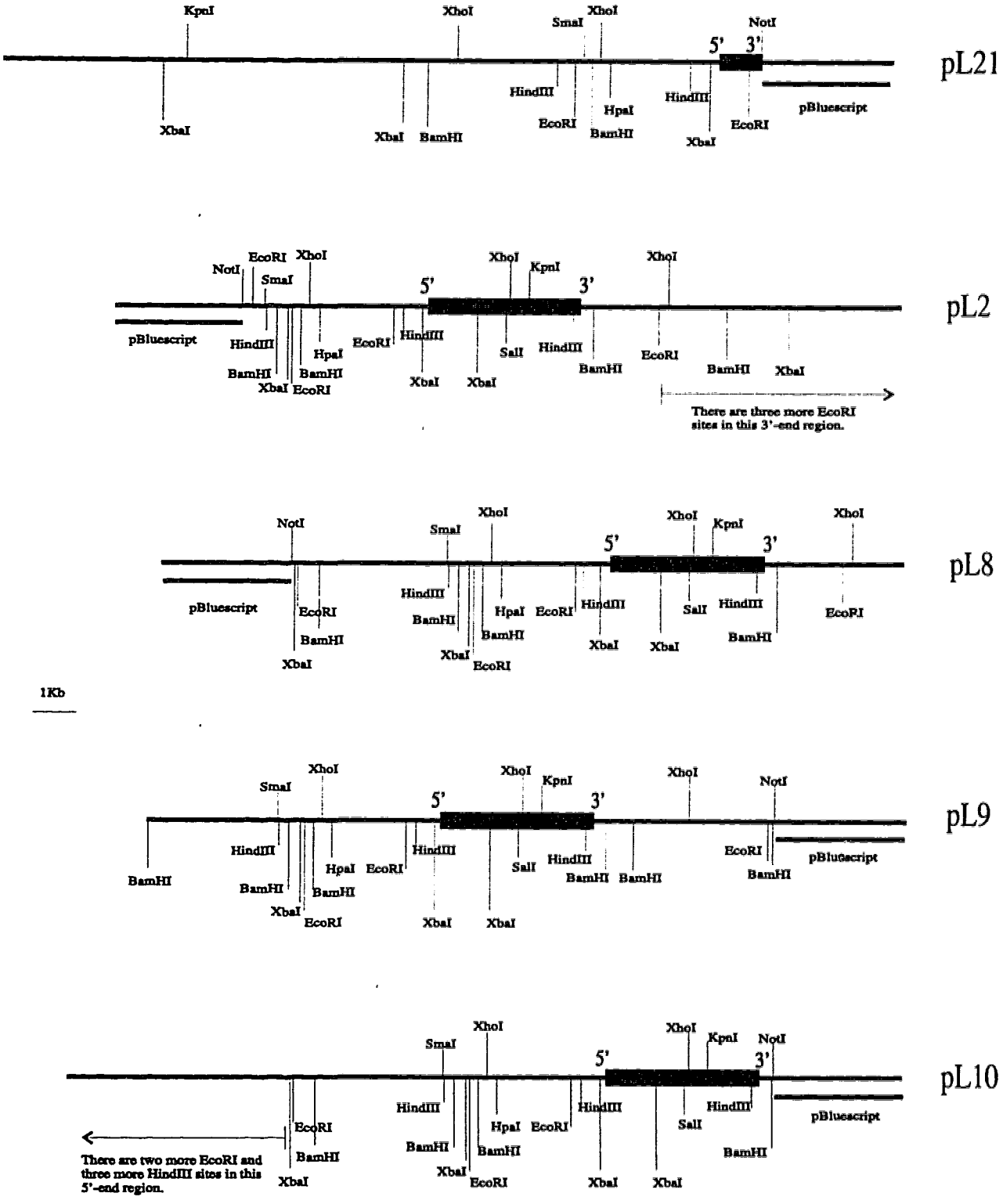


Figure 1-5. PCR screening for clone 91 sequences. The primers used in the PCR reactions were KWO1 and KWO3 (Fig1-1). The templates used were as follows: in lane C, 91R91, used as positive control: in lane 1, 2, 3, and 4 are phage suspensions of λ21, λ2, λ8, λ9, and λ10 respectively. The fragment produced by PCR is predicted to be 143 bp. M: molecular weight marker, the sizes of bands are indicated on the left side.

Figure 1-6. Restriction maps of five α_{2u}–globulin clones. The bold lines indicate the coding region of α_{2u}-globulin gene. The genomic sequences and the vector sequences (pBluescript) are separated by Not I sites.


pL21
KpnI
XhoI
SmaI
XhoI
NotI
5'
3'
HindIII
HindIII
HpaI
pBluescript
XbaI
XbaI
BamHI
EcoRI
BamHI
XbaI
EcoRI
pL2
NotI
EcoRI
SmaI
XhoI
XhoI
KpnI
XhoI
5'
3'
pBluescript
HindIII
HpaI
EcoRI
HindIII
SalI
HindIII
EcoRI
BamHI
XbaI
BamHI
BamHI
XbaI
EcoRI
XbaI
XbaI
BamHI
XbaI
There are three more EcoRI sites in this 3'-end region.
pL8
NotI
SmaI
XhoI
XhoI
KpnI
XhoI
5'
3'
pBluescript
EcoRI
HindIII
HpaI
EcoRI
HindIII
SalI
HindIII
EcoFI
BamHI
BamHI
BamHI
BamHI
XbaI
XbaI
XbaI
XbaI
EcoRI
BamHI
1Kb
pL9
XhoI
SmaI
XhoI
KpnI
XhoI
NotI
5'
3'
BamHI
HindIII
HpaI
EcoRI
HindIII
SalI
HindIII
EcoRI
pBluescript
BamHI
BamHI
BamHI
BamHI
BamHI
BamHI
XbaI
EcoRI
XbaI
XbaI
pL10
XhoI
SmaI
XhoI
KpnI
NotI
5'
3'
HindIII
HpaI
EcoRI
HindIII
SalI
HindIII
pBluescript
EcoRI
BamHI
BamHI
BamHI
BamHI
BamHI
XbaI
XbaI
EcoRI
XbaI
XbaI
There are two more EcoRI and three more HindIII sites in this 5'-end region.

```
                                                                pL21  -204
CTGGTTCCAG ATGGCCCTCT AGAACACCCA CTGTTTTTCT TGGGAATATG
                    :::: :::::::::: :::::::::: ::::::::::
                    CTCT AGAACACCCA CTGTTTTTCT TGGGAATATG
                  -238                                  91R91  -204


                                                               pL21  -154
TTTTGCGAAA TGTATGAGTG ATAGAATCAA TCCATAGGAG ATGACATCGC
:::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
TTTTGCGAAA TGTATGAGTG ATAGAATCAA TCCATAGGAG ATGACATCGC
                                                             91R91  -154


                                                               pL21  -104
CAAGTTTCAA AAGGGCAGGA ACAATCCTTG GCTTCACATC AGTACATGAG
:::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
CAAGTTTCAA AAGGGCAGGA ACAATCCTTG GCTTCACATC AGTACATGAG
                                                           91R91  -104


                                                                pL21  -54
AAAACATTCC ACAAAGCCTG AAGGATGGAG GCCCATATGA GAAGGAAAAA
:::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
AAAACATTCC ACAAAGCCTG AAGGATGGAG GCCCATATGA GAAGGAAAAA
                                                           91R91  -54


                                                                pL21   -4
AAAACACTCA AACCCAGAGA GAGTATAAAG ACGAGCAAAG TGCTGGAGGT
:::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
AAAACACTCA AACCCAGAGA GAGTATAAAG ACGAGCAAAG TGCTGGAGGT
                                                           91R91   -4

   |+1
                                                               pL21  +47
GGAGTGTGGG CACCATCAGC AGAGGGATTG TCCCGACAGA GAGGCAATTC
:::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
GGAGTGTGGG CACCATCAGC AGAGGGATTG TCCCGACAGA GAGGCAATTC
                                                           91R91  +47


                                                               pL21  +97
TATTCCCTAC CAACATGAAG CTGTTGCTGC TGCTGCTGTG TCTGGGCCTG
:::::::::: ::::::
TATTCCCTAC CAACAT
      91R91  +63
```

Figure1-7. Comparison of the 5'-upstream sequences of the α_{2u}-globulin genes in pL21 and 91R91. The sequences are numbered with respect to the transcription initiation site, +1.

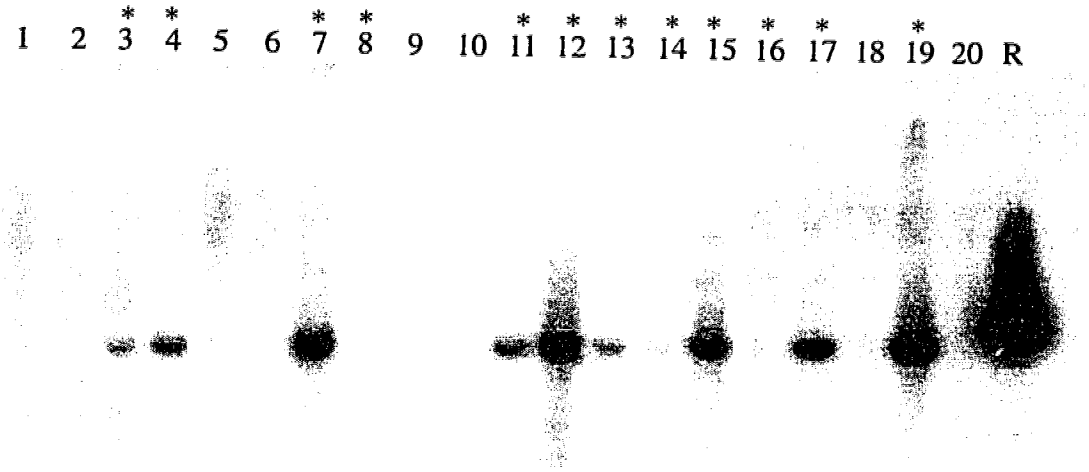


Figure 1-8. Southern blot analysis of cell lines transfected with 91RX2 (Fig.1-4). Each cell line was derived from a single colony. Genomic DNA (20μg) from each cell line was digested with HindIII and fractionated on 0.8% agarose gel, and the blot was hybridized with $4x10^5$ cpm/ml of the radioactively labelled 4.1 kb HindIII fragment from 91R91 (Fig. 1-3). This fragment was also used in this Southern blot, marked R, as a positive control. The numbers on the top designate the different cell lines. Those marked (*) were tested for the inducibility of α_{2u}-globulin gene expression by dexamethasone.

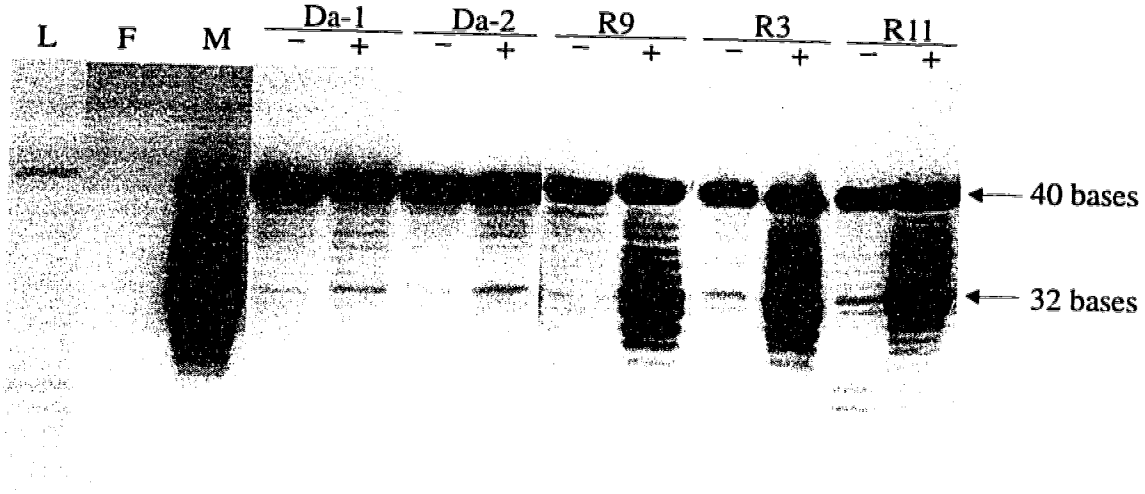


Figure1-9. Detection of α_{2u}-globulin RNA by the S1 protection assay. Two independent cell lines, Da-1 and Da-2, obtained from transfection with 91Da (see Fig. 1-4) and three independent cell lines from transfection with 91R91, R9, R3 and R11, were treated with dexamethasone (+), or without dexamethasone (-) for nine days as described in Materials and Methods.The analysis was carried out with 25 µg of total RNA from these cell lines, as well as RNA from L-cells: L, liver RNA from an adult female rat: F, and liver RNA from an adult male rat: M. The protected 32 base fragment was cut out to quantitate RNA specific to α_{2u}-globulin.

Construct	91Da	91R91	91RX3	91RX2	91RX4
Length of 5'-upstream sequence	226 bp	762 bp	2.7 kb	3.2 kb	6.3 kb
Number of cell lines tested	3	5	6	12	10
Number of transcribable lines	3	5	5	5	6
Range of induction levels (fold)	2.7-7.0	16-44	17-45	6.0-38	5.0-24
Average level of induction	4.24	23.51	23.94	21.76	16.16

Table 1-1. Dexamethasone induction of the α_{2u}-globulin gene in cell lines transfected with the constructs listed in Fig.1-4. The induction of all cell lines with dexamethasone was performed as described in the Materials and Methods, and α_{2u}-globulin RNA was quantitated by S1 protection. Cell lines that did not yield a significant level of the 40 base fragment were considered non-transcribable.

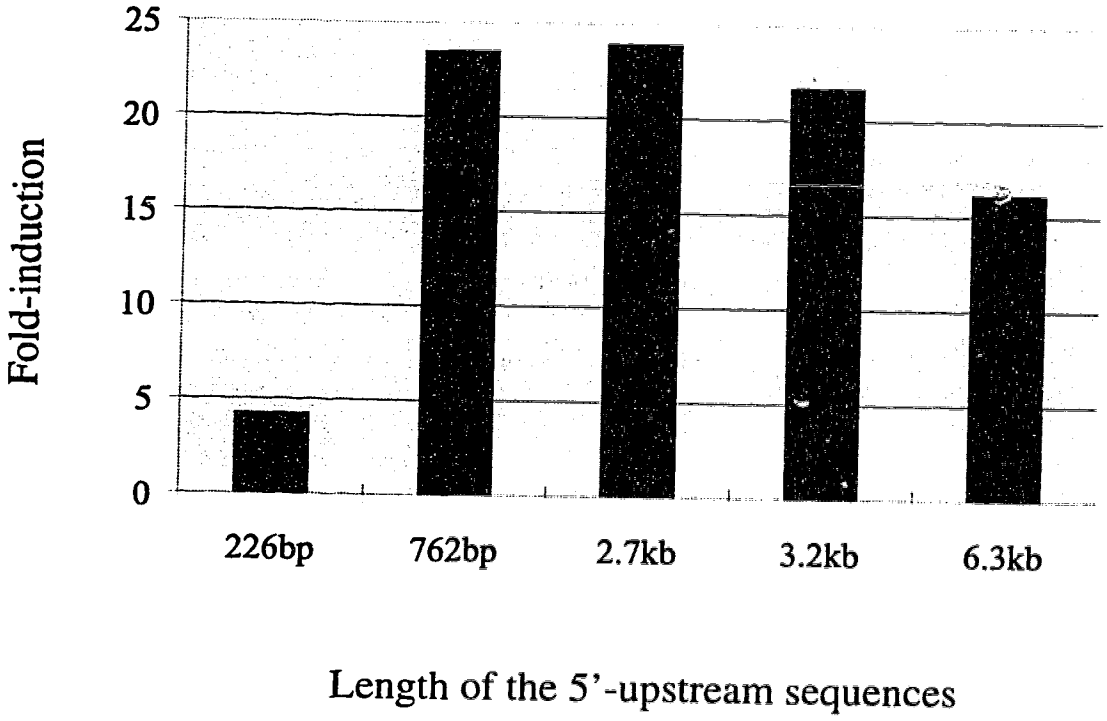


Figure 1-10. Effects of the 5'-upstream sequences on the induction of α_{2u}-globulin RNA by dexamethasone.The average induction of each construct shown in Table1-1 was replotted in this chart.

Figure 1-11. 5'-upstream sequences of clone 91. Sequences are numbered from the transcription initiation site, +1. GRE: possible glucocorticoid response element. The consensus GRE is AGAACANNNTGTACC (Truss and Beato 1993).

-719 TCGGAAGAC CATTCCTGAT -701

AATCTTTTTA AATTAAGATT TATTATTTTT ATGCATATGT GCCTGAATGA -651

GTGAGTTCAT ATGTACCACA TGTGTACAGG AGATCACAGG GGCCAAAAGA -601
GRE

GGGTCATTTC CTGTGACTGG AGCTAGAGTC AGTTGTGAAA TGATATGTGA -551

GTGCTGGGAA TTGAACCTGG GTCCTCAGCA AGAACAGGTT TCAAAGAATC -501
GRE

TGACTCCTTC TTTTGACCTC CTTTTGACTT CCTTGGCCCC AGGTGTGTGC -451

ATGGTACACA TGCAAAACCA ATGCTCATAC GCATGAAATG TGAATGAATC -401

TTTTCAAAGA AAACCCAAGT AATTCAGCTT CTTCCACACT CCACTCAAGT -351

CTTGAAGCAA AGATTCTCTC CATGCTTGAC CTTCCAGTTC AGTACCCACC -301

CACTCCATAA CTGGTTTTCT GATTCTAAGT CAGATCCAAT GTTGCATCTG -251

GTTCCAGATG GCCCTCTAGA ACACCCACTG TTTTTCTTGG GAATATGTTT -201

TGCGAAATGT ATGAGTGATA GAATCAATCC ATAGGAGATG ACATCGCCAA -151

GTTTCAAAAG GGCAGGAACA ATCCTTGGCT TCACATCAGT ACATGAGAAA -101

ACATTCCACA AAGCCTGAAG GATGGAGGCC CATATGAGAA GGAAAAAAAA -51

ACACTCAAAC CCAGAGAGAG TATAAAGACG AGCAAAGTGC TGGAGGTGGA -1

+1
GTGTGGGCAC CATCAGCAGA GGGATTGTCC CGACAGAGAG GCAATTCTAT +50

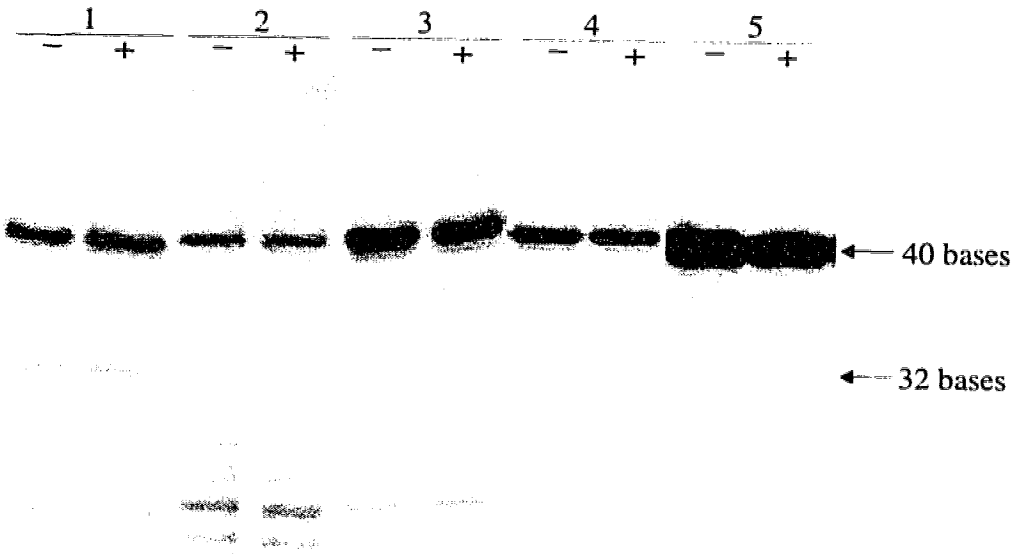


Figure 1-12. Detection of α_{2u}-globulin RNA by the S1 protection assay. Number 1 and 2 are cell lines transfected with DNA from pL9 that contains 2.7 kb 5'-upstream sequences, number 3 is from a pL8 transfectant containing 2.7 kb 5'-upstream sequences, number 4 and 5 are from transfectants of pL2 containing 762 bp 5'-upstream sequence. In addition to these 5'-upstream sequences, all constructs used in transfection contain the coding region and 400 bp 3'-downstream region of α_{2u}-globulin gene, and RX vector sequences. Cells were treated with dexamethasone (+), or without dexamethasone (-) for nine days as described in Materials and Methods.The analysis was carried out with 25 µg of total RNA from these cell lines. The protected 32 base fragment was cut out to quantitate RNA specific to α_{2u}-globulin.

```
91R91
pL2       ATATCGAATTCTTTATTTCCCCACATCAAACACTATTTTATGTTTCTAGT
pL8       ---------------------------------------------------
pL9       ---------------------------------------------------
pL10      ---------------------------------------------------
          -1047                                          -998

91R91
pL2       TCCAGGGTAAATACAATTTATATGGATACAAAAAATATTTATAGCTGTGT
pL8       ---------------------------------------------------
pL9       ---------------------------------------------------
pL10      ---------------------------------------------------
                                                         -948

91R91
pL2       GTCTTTGTAAGCAGACTCGACATCATTTCTACTTGGACCTCCATCAGCAA
pL8       ---------------------------------------------------
pL9       ---------------------------------------------------
pL10      ---------------------------------------------------
                                                         -898

91R91
pL2       ATTTGACTTTGAACAATACCTTTGTTTTCTTTAATGATGAGATTATTTCC
pL8       ---------------------------------------------------
pL9       ---------------------------------------------------
pL10      ---------------------------------------------------
                                                         -848

91R91
pL2       AGTCAGTTAAAGTCCAACGTTCTCTGAGGTAAACAGCTGAAGGTGTTTAT
pL8       ---------------------------------------------------
pL9       ---------------------------------------------------
pL10      ---------------------------------------------------
                                                         -798
```

Figure 1-13. Comparison of the 5'-upstream sequences of the α_{2u}-globulin gene in five clones: 91R91 (clone 91), pL2, pL8, pL9 and pL10. Sequences are numbered according to the transcription initiation site, +1.

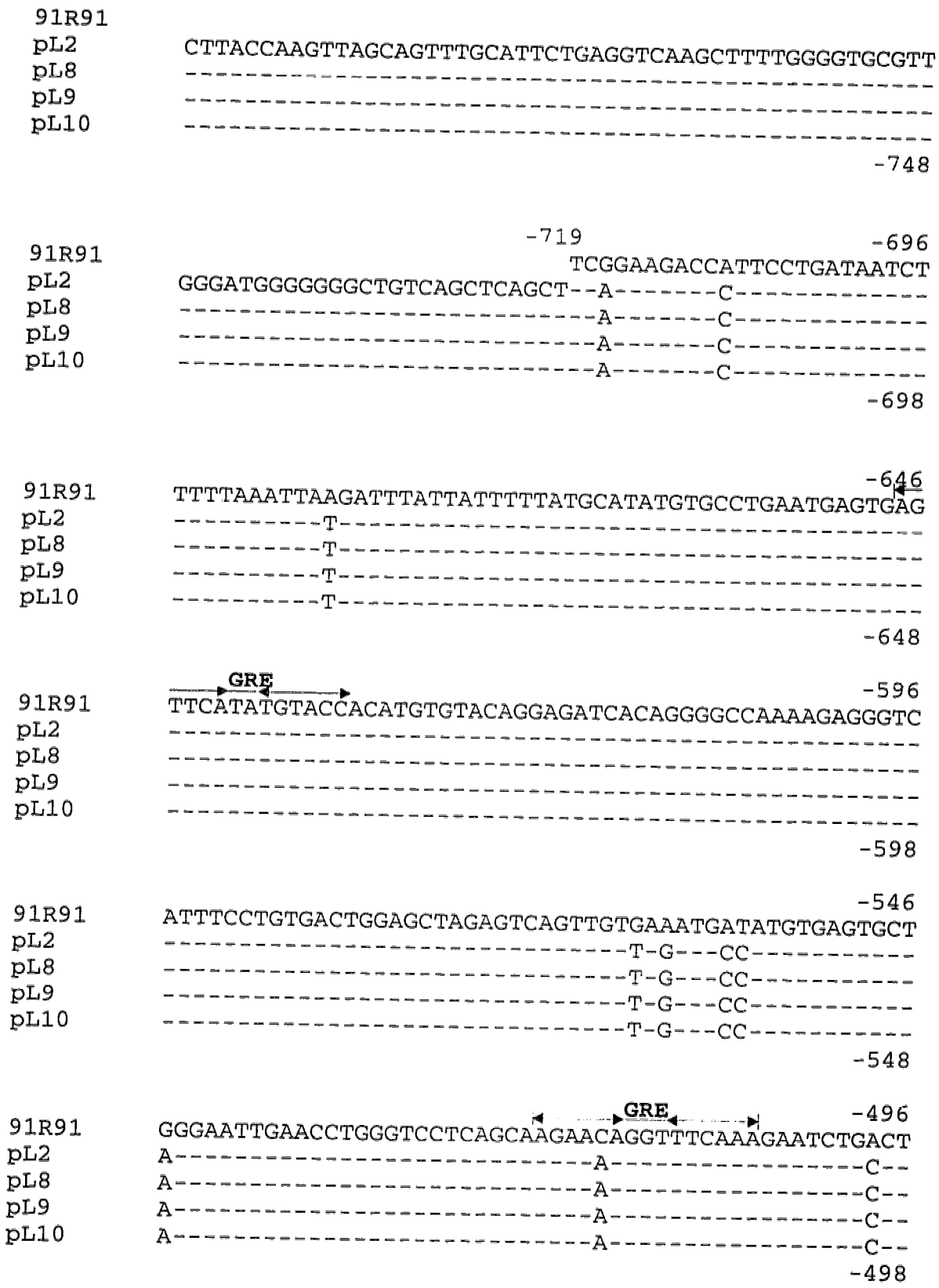


Figure 1-13. Continued. GRE: possible glucocorticoid receptor binding site in clone 91.

```
                                                                    -446
91R91       CCTTCTTTTGACCTCCTTTTGACTTCCTTGGCCCCAGGTGTGTGCATGGT
pL2         ----T---  ---------C------T----------------------
pL8         ----T---  ---------C------T----------------------
pL9         ----T---  ---------C------T----------------------
pL10        ----T---  ---------C------T----------------------
                                                                    -449


                                                                    -396
91R91       ACACATGCAAAACCAATGCTCATACGCATGAAATGTGAATGAATCTTTTC
pL2         ----------------------------------T---------------
pL8         ----------------------------------T---------------
pL9         ----------------------------------T---------------
pL10        ----------------------------------T---------------
                                                                    -399

                                                                    -346
91R91       AAAGAAAACCCAAGTAATTCAGCTTCTTCCACACTCCACTCAAGTCTTGA
pL2         ---------------------------------T----------------
pL8         ---------------------------------T----------------
pL9         ---------------------------------T----------------
pL10        ---------------------------------T----------------
                                                                    -349

                                                                    -296
91R91       AGCAAAGATTCTCTCCATGCTTGACCTTCCAGTTCAGTACCCACCCACTC
pL2         ---------A----T---------------------------G-------
pL8         ---------A----T---------------------------G-------
pL9         ---------A----T---------------------------G-------
pL10        ---------A----T---------------------------G-------
                                                                    -299

                                                                    -246
91R91       CATAACTGGTTTTCTGATTCTAAGTCAGATCCAATGTTGCATCTGGTTCC
pL2         ---------C----------C-------------A-G---C-----A--T
pL8         ---------C----------C-------------A-G---C-----A--T
pL9         ---------C----------C-------------A-G---C-----A--T
pL10        ---------C----------C-------------A-G---C-----A--T
                                                                    -249

                                                                    -196
91R91       AGATGGCCCTCTAGAACACCCACTGTTTTTCTTGGGAATATGTTTTGCGA
pL2         ------------------------------------------------T--
pL8         ------------------------------------------------T--
pL9         ------------------------------------------------T--
pL10        ------------------------------------------------T--
                                                                    -199
```

Figure 1-13. Continued.

```
                                                                    -146
91R91     AATGTATGAGTGATAGAATCAATCCATAGGAGATGACATCGCCAAGTTTC
pL2       ------------G-A--------------------GG-T---------G
pL8       ------------G-A--------------------GG-T---------G
pL9       ------------G-A--------------------GG-T---------G
pL10      ------------G-A--------------------GG-T---------G
                                                          -149

                                                                     -96
91R91     AAAAGGGCAGGAACAATCCTTGGCTTCACATCAGTACATGAGAAAACATT
pL2       ----------------------------------------------------------
pL8       -------------------------
pL9       ----------------------------------------------------------
pL10      ----------------------------------------------------------
                                                                   -99

                                                                  -49
91R91     CCACAAAGCCTGAAGGATGGAGGCCCATATGAGAAGGAAAAAAAA    AC
pL2       ------------G-T-A---------C-----------------ACAC-
pL8
pL9       ------------G-T-A---------C-----------------ACAC-
pL10      ------------G-T-A---------C-----------------ACAC-

                                                                 +1
91R91     ACTCAAACCCAGAGAGAGTATAAAGACGAGCAAAGTGCTGGAGGTGGAGT
pL2       ----G--------------------T---------C--T----------
pL8
pL9       ----G--------------------T---------C--T----------
pL10      ----G--------------------T---------C--T----------

                                                                  +52
91R91     GTGGGCACCATCAGCAGAGGGATTGTCCCGACAGAGAGGCAATTCTATTC
pL2       ----------------------------------------------------------
pL8
pL9       ----------------------------------------------------------
pL10      ----------------------------------------------------------
```

Figure 1-13. Continued. +1: the transcription initiation site.

```
                                                                    -646
91        TTTTAAATTAAGATTTATTATTTTTATGCATATGTGCCTGAATGAGTGAG
RAP01     ---------------------------------------------------
#2        ----------T----------------------------------------
RAO01     --------A-C-------------------------------C---------

                GRE                                         -596
91        TTCATATGTACCACATGTGTACAGGAGATCACAGGGGCCAAAAGAGGGTC
RAP01     ---------------------------------------------------
#2        ---------------------------------------------------
RAO01     --------------------T-----------A--A-------------

                                                            -546
91        ATTTCCTGTGACTGGAGCTAGAGTCAGTTGTGAAATGATATGTGAGTGCT
RAP01     ---------------------------- ---G---CC-----------
#2        ------------------------------T-G---CC-----------
RAO01     ---------------------------- ---G---CC----------G

                                    GRE                     -496
91        GGGAATTGAACCTGGGTCCTCAGCAAGAACAGGTTTCAAAGAATCTGACT
RAP01     A----------T-------------------------------A------
#2        A--------------------------A-----------------C--
RAO01     A--------------------------A--------------------

                                                            -446
91        CCTTCTTTTGACCTCCTTTTGACTTCCTTGGCCCCAGGTGTGTGCATGGT
RAP01     ---C--------------C--G----------------------------
#2        ----  -------------C------T-----------------------
RAO01     ----  C---C--A-----C------T-----------A------------

                                                            -396
91        ACACATGCAAAACCAATGCTCATACGCATGAAATGTGAATGAATCTTTTC
RAP01     ---------------------------------------------------
#2        ----------------------------------T----------------
RAO01     -------------------------A---------     -----G----T

                                                            -346
91        AAAGAAAACCCAAGTAATTCAGCTTCTTCCACACTCCACTCAAGTCTTGA
RAP01     ----------------------TT-------------------------
#2        ------------------------------T-------------------
RAO01     ---------------------------------------------------
```

Figure 1-14. Comparison of the 5'-upstream sequences of the α_{2u}-globulin gene in clones: 91, #2, RAP01 and RAO01 (Van Dijck et al., 1993). Sequences are numbered according to the transcription initiation site. GRE: possible glucocorticoid response element.

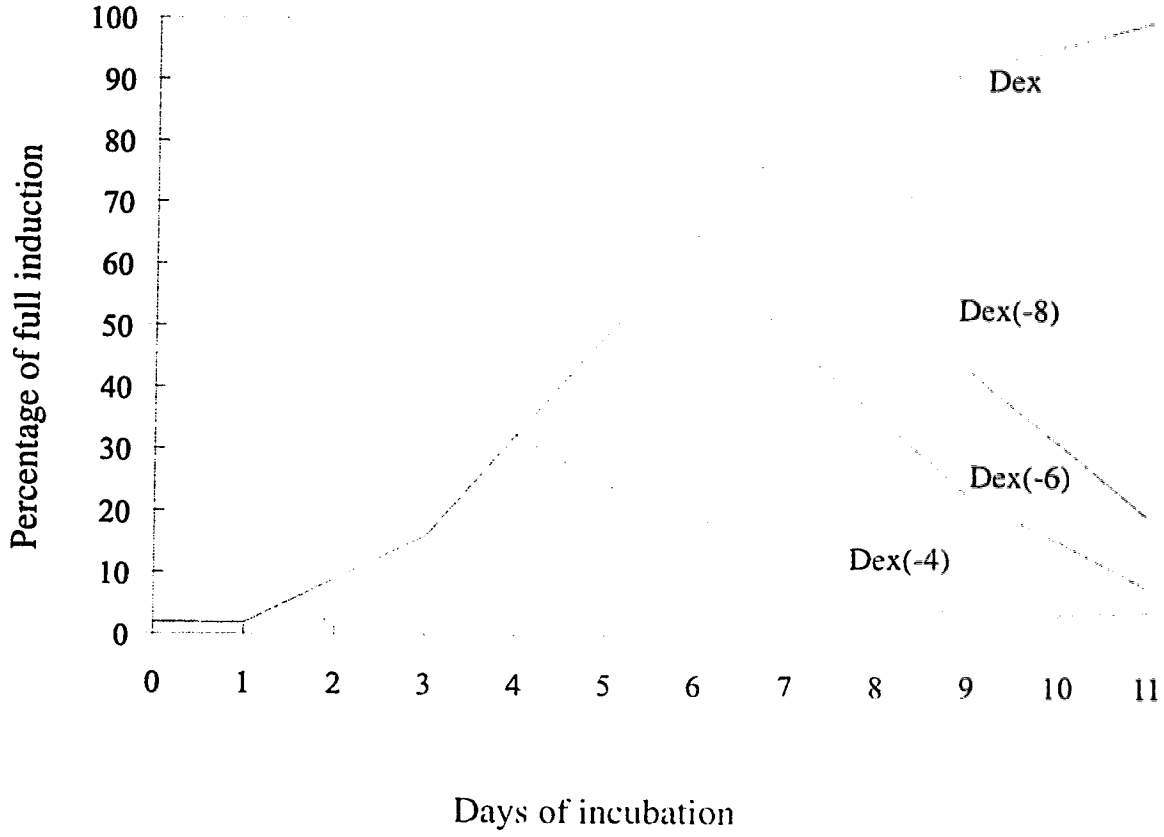


Figure 1-15. Effect of dexamethasone withdrawal on the transcription of α_{2u}-globulin RNA from clone 91. The experiment was conducted as described in Materials and Methods. Dexamethasone was withdrawn from the medium on the 4th day of induction: Dex(-4), on the 6th day of induction: Dex(-6), and on the 8th day of induction: Dex(-8). The amount of α_{2u}-globulin mRNA was quantitated by S1 protection. Dex: control group with continued dexamethasone treatment, the fold-induction of this group on the 11th day was used as 100% induction in the graph.

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Chapter 2

The effect of estrogen on the expression of α_{2u}-globulin gene, clone 91, in mouse L-cells

Introduction

Of all the hormones that affect the transcription of α_{2u}-globulin genes in rats, the action of estrogen is the most complex. Some of the early studies were conducted with castrated female rats to remove any possible inhibitors for the expression of this gene. Following an initial exposure of castrated female rats to estrogenic hormones, an increase in the hepatic concentration of α_{2u}-globulin was observed, which could be blocked with cycloheximide. In this initial induction of α_{2u}-globulin gene, estrogen is just as effective as androgen. But this induction by estrogen is gradually lost after continued daily treatment (Roy 1977). Daily treatment of mature male rats with estrogenic hormones brings about a large decrease in the hepatic synthesis of α_{2u}-globulin, a 50% decrease in 4 days and complete suppression of α_{2u}-globulin synthesis in 8 days. This continued treatment with estrogen eventually produces a two week long quiescent period, during which the α_{2u}-globulin gene does not response to the inductive hormone, androgen (Roy et al., 1975). Although estrogen can decrease the hepatic level of androgen receptor (Kurtz et al., 1976), the suppression of α_{2u}-globulin gene transcription by estrogen did not appear to be via this mechanism (Roy 1979; Kurtz and Feigelson 1978).

The regulation of gene expression by estrogen-activated receptor has been studied in many systems. In most cases, the estrogen receptor binds to the estrogen response

element (ERE) in the promoter region of the regulated gene. The consensus sequence of the ERE in most of the estrogen regulated genes is a palindromic sequence with a half site of GGTCA separated by a 3 bp spacer. Sequences of TCAGGTCA or TTAGTTCA are also functional as estrogen receptor binding sites (Mangelsdorf et al., 1995). Upon binding to the promoter region, the estrogen receptor interacts with the transcription initiation complex to alter the transcription of the gene. The mechanism underlying this interaction is not clear. The requirement of protein synthesis in some cases implied that factors other than the estrogen receptor and the transcription initiation complex are involved in this process. Several nuclear factors that can interact with the estrogen receptor have been cloned and identified. One of these factors, ERAP160, exhibited binding specificity to estrogen-activated receptor (Halachmi et al., 1994), implicating a direct involvement in the estrogen-regulated gene transcription. Nuclear factor RIP140 was also found to interact specifically with the activation domain of the estrogen receptor in an estrogen dependent manner (Cavailles et al., 1995), which also suggested this nuclear factor is a potential co-activator of estrogen-regulated gene transcription.

Although the regulation of rat α_{2u}-globulin genes by estrogen *in vivo* has been evident for over two decades, there is little known about how this occurs at the molecular level. The only suggestion came from *in vitro* studies with clone RAO01, which showed that a fragment from this clone, from -606 bp to -575 bp, has significant binding affinity with the estrogen receptor. An estrogen receptor binding site, in which a 7 bp spacer separates the two half sites, was located in this fragment (Van Dijck and Verhoeven 1992). However, its functional significance *in vivo* is not yet known.

In the previous chapter it was shown that clone 91 is induced by dexamethasone in mouse L-cells. Therefore, these cells have the factors required for the expression of this α_{2u}-globulin gene. Since *in vivo* studies have shown that estrogen can initially induce the expression of α_{2u}-globulin in castrated female, and suppress its expression in male upon continued treatment, the mouse L-cell system was also used to explore whether

estrogen would induce the expression of clone 91 or whether it would suppress the expression of clone 91 induced by dexamethasone in this system.

Materials and Methods

Plasmid constructs. The construction of plasmids 91RX4, 91RX2, 91RX3, 91R91 and 91Da is described in Chapter 1.

Transfection of mouse L cells with ΔHER. ΔHER is a plasmid derived from inserting a 2kb fragment containing human estrogen receptor cDNA into an EcoRI cloning site in the expression vector p91023(B) (Wong et al., 1985). The human estrogen receptor cDNA in this plasmid is under the control of an SV40 promoter (Dr. Tsai, personal communication). Plasmid p220.2 contains a hygromycin resistant gene (Dr. McArdle, personal communication)

Mouse L tk^- $aprt^-$ cells (Weglier et al., 1979) were transfected by a modified method of Ausubel et al., (1995). One day before transfection, confluent cells from one 100 mm plate were split 1: 10 in Dulbecco modified minimum essential medium (DMEM) plus 10% calf serum. Two hours before transfection, the medium was replaced with 9 ml of the same medium. To transfect, cells in each plate were treated with 1 ml of calcium phosphate precipitate containing 15 μg of ΔHER, 1 μg p220.2 and 15 μg of mouse L-cell carrier DNA. After 16 hours, the cells were washed twice with PBS and fed with medium DMEM plus 10% calf serum. Cells were incubated for another 48 hours, then the medium was replaced with selective medium which contained 400 μg/ml of Hygromycin B. After two weeks of selection, single colonies were isolated, and further cultivated as cell lines. Cell lines were examined for the presence of the human estrogen receptor gene by Southern blotting, and they were also examined for the presence of nuclear localized estrogen receptor with estrogen receptor antibody by the Medical Pathology Laboratory at the Cross Cancer Institute. An established estrogen receptor

positive cell line E2 was used in the transfection with constructs containing the α_{2u}-globulin gene clone 91.

Cell line E3-a was established by transfecting L-a cells, which had been previously transfected with 91R91, with ΔHER as described above. Line E3-a was also tested positive for the presence of estrogen receptor using Southern blotting and antibody detection.

Transfections with α_{2u}-globulin gene constructs were conducted as described in the Materials and Methods of Chapter 1, except the cells used here were E2 cells. Isolated cell lines were examined for the presence of α_{2u}-globulin gene by Southern blotting, and the presence of α_{2u}-globulin RNA in these cell lines was examined by RNA S1 protection assays.

DNA extraction and Southern hybridization. Tissue culture cell genomic DNA was extracted according to Ausubel et al., (1995). For Southern analysis, 20 μg of DNA from each cell line was digested with restriction enzymes (BRL), fractionated in a 0.8% agarose gel and transferred onto a Zeta-probe nylon membrane (Bio-Rad Laboratories) according to manufacturer's instructions. The probe used in Southern blots for ΔHER transfected cells was a 2 kb EcoRI fragment from ΔHER that contains human estrogen receptor cDNA labeled with α-^{32}P-dATP. The probe used in Southern analysis to detect α_{2u}-globulin sequences in transfected cells was the 4.2 kb HindIII fragment from 91R91 (see Fig. 1-3) labeled with α-^{32}P-dATP.

Induction of α_{2u}-globulin RNA with hormones. Cells containing α_{2u}-globulin constructs were fed with DMEM + 10% calf serum medium and split 1:8 the day before induction. Two plates of cells were used in each sample group. In control groups, cells were fed with DMEM+ 10% calf serum. In the induced groups, cells were fed with DMEM + 10% calf serum + hormone. In inductions with estrogen, 2.75×10^{-5} M of 17β-estrodiol was used. In inductions with dexamethasone, 1×10^{-6} M of dexamethasone was

used. The medium in all cell cultures was replaced once every two days and the cells were harvested on the 9th day of induction for RNA isolation.

For the dosage dependency analysis of estrogen induction, one group of control cells was fed with DMEM + 10% calf serum, five induction groups were fed with DMEM + 10% calf serum plus $0.55x10^{-5}$ M, $1.1x10^{-5}$ M, $1.65x10^{-5}$ M, $2.2x10^{-5}$ M and $2.75x10^{-5}$ M of 17β-estrodiol respectively. Cells were fed once every 2 days and were harvested on the 9th day of induction.

The kinetics of estrogen induction was examined by feeding E3-a cells with fresh medium containing $2.75x\ 10^{-5}$ M of 17β-estrodiol on the 0, 2nd, 4th, 6th, 8th, and 10th day. Two plates of cells were harvested on the 1st, 3rd, 5th, 7th, 9th, and 11th day of induction. Cells in two control plates were fed every two days with unsupplemented fresh medium and harvested on the 7th day. RNA was isolated from these cells and the quantity of α_{2u}-globulin RNA was determined by S1 protection.

RNA isolation and S1 protection assay. The isolation of total RNA from cells and the quantitation of α_{2u}-globulin RNA by S1 protection were described in the Materials and Methods of Chapter 1.

Results.

Establishment of L-cell lines which express estrogen receptors. Prior to choosing mouse L-cells for the exploration of estrogen effects on the expression of the α_{2u}-globulin gene, several cell lines which express the estrogen receptor, including human MCF-7 cells, mouse T3 cells and mouse T4 cells, were tested for their suitability. The α_{2u}-globulin gene construct, 91R91 (see Fig. 2-7), was transfected into T3 or T4 cells, but in neither cell line was the gene inducible by dexamethasone. 91R91 transfected MCF-7

cells never survived the selection in G418. It is possible that α_{2u}-globulin is toxic to these cells even at low level, since humans have no homologues to the gene family.

L-cells were transfected with ΔHER and single colonies were isolated and further cultivated as cell lines. Southern blot analysis showed some of the cell lines contain integrated estrogen receptor gene sequences (Fig.2-1). This result was further corroborated by anti-estrogen receptor antibody localizations which demonstrated estrogen receptor positive nuclei in E2 (data not shown). This cell line was chosen for transfections with clone 91 constructs discussed later in this chapter.

Cell line L-a, which was transfected with 91R91 and showed inducibility of α_{2u}-globulin by dexamethasone, was also transfected with ΔHER. One cell line, E3-a, which showed positive integration of estrogen receptor on the Southern blot (Fig.2-1 lane 8) and a positive antibody response, was established and used to characterize the parameters of induction of α_{2u}-globulin by estrogen.

α_{2u}-Globulin RNA, in clone 91, is induced by estrogen. Independent cell lines derived from the transfection of E2 with 91R91, which carried the coding region and 762 bp of 5'-upstream sequences of clone 91, were confirmed for the presence α_{2u}-globulin sequences by Southern blotting and the inducibility of α_{2u}-globulin RNA by dexamethasone (data not shown). In four of these cell lines, E2-R11, E2-R9, E2-R8, E2-R10, and E3-a as well, estrogen induction of the transcription of α_{2u} globulin RNA was observed (Fig. 2-2). The S1 protection assay showed that the α_{2u}-globulin RNA in these cell lines increased 5-15 fold following 9 days of estrogen treatment.

Further testing with cell line E3-a showed that this induction of α_{2u}-globulin gene was dependent upon the concentration of estrogen (Fig. 2-3). The fold-induction increases as the concentration of 17β-estrodiol rises until the concentration reaches 2.2×10^{-5} M, where α_{2u}-globulin RNA levels start to plateau.

The kinetics of estrogen induced α_{2u}-globulin transcription in E3-a cells (Fig. 2-4) showed a lag period of 2 to 3 days, which is similar to what we observed during dexamethasone induction of α_{2u}-globulin gene in L-cells (see Fig 1-15 or Addison and Kurtz, 1986). Following the lag period, the α_{2u}-globulin RNA increases at a linear rate until the 9th day of treatment, where the level of α_{2u}-globulin RNA becomes relatively steady. The presence of a lag period suggests that the induction by estrogen may be a secondary response and require the synthesis of other transcription factor(s). This requirement of protein synthesis has been confirmed by the response of estrogen induction to cycloheximide treatment in E3-a cells (Fig. 2-5), in which the administration of 0.05 μg/ml cycloheximide completely blocked the induction of α_{2u}-globulin transcription. The measurement of cycloheximide sensitivity was conducted by quantitating the amount of the α_{2u}-globulin RNA in 25 μg of total cell RNA. The level of 17β-estrodiol induced transcription in cells treated with 0.05 μg/ml cycloheximide is equivalent to the level of transcription in cells without 17β-estrodiol. The dramatic decrease of the relative amount of α_{2u}-globulin RNA in the total cell RNA indicates that the transcription of the α_{2u}-globulin gene induced by estrogen is more sensitive to the inhibition of protein synthesis than the overall transcription in the cells, suggesting specific protein synthesis is required for estrogen induction.

Synergistic effect of estrogen and glucocorticoid on α_{2u}-globulin gene transcription. *In vivo* studies have shown that continued daily treatment with estrogen for 8 days suppresses the expression of α_{2u}-globulin gene in male rat livers. To investigate the effect of estrogen on the dexamethasone induced transcription of the α_{2u}-globulin gene in mouse L-cells, 17β-estrodiol was added at the same time as dexamethasone. In two independent cell lines, E2-R8 and E2-R11, in which the transcription of the α_{2u}-globulin gene was inducible by either 17β-estrodiol or dexamethasone, the treatment with both 17β-estrodiol and dexamethasone for 9 days produced a much higher level of α_{2u}-globulin gene transcription than that produced by treating with either of the hormones alone (Fig. 2-6). In fact the level of induction by both hormones is higher than the sum of

the levels of induction produced by these two hormones individually, indicating a synergistic effect was produced.

5'-upstream sequences responsible for the estrogen induced transcription of α_{2u}-globulin gene. In order to examine the 5'-upstream sequences required for the induction of α_{2u}-globulin gene by estrogen, and the effect of 5'-upstream sequences on the synergistic effect produced by estrogen and dexamethasone treatment, constructs which contain different lengths of 5'-upstream sequences of the α_{2u}-globulin gene from clone 91 (Fig. 2-7) were transfected into E2 cells. Three to five independent cell lines from the transfectants of each construct, in which the α_{2u}-globulin gene was found to be inducible by dexamethasone, were tested for the inducibility by 17β-estrodiol or both hormones (Fig. 2-8 to Fig. 2-12). The results summarized in Figure 2-13 show that cells transfected with 91R91, which contains 762 bp of 5'-upstream sequence, have a significantly higher estrogen induction level than those with any of the other constructs. The average level of induction by estrogen in these cell lines is 7.6-fold. The removal of the -762 bp to -220 bp upstream region or the addition of 5'-upstream sequences beyond -762 bp reduces the inducibility of α_{2u}-globulin gene by estrogen. The synergistic effect of estrogen and dexamethasone action on the transcription of α_{2u}-globulin gene in clone 91 seems more dependent on the inducibility of α_{2u}-globulin gene by estrogen. When the induction of the α_{2u}-globulin gene by estrogen is lower, the induction by both hormones is more or less additive rather than synergistic.

The decrease of inducibility of the α_{2u}-globulin gene in cells that contain 220 bp upstream sequences, compared with that in cells containing 762 bp of 5'-upstream sequences, indicates that the sequence between -767 bp and -226 bp is important for the estrogen induction. Sequencing analysis of this entire region revealed the presence of a sequence that is highly homologous to the estrogen receptor binding site (ERE), which is 60 bp upstream of the glucocorticoid receptor binding site (GRE) discussed in Chapter 1 (Fig.2-14). The sequence around the ERE site, -606 bp to -575 bp, is identical to the sequence in clone RAO01, which showed binding affinity to the estrogen receptor *in vitro*

(Van Dijck and Verhoeven 1992). Thus the ERE at -590 bp is most probably responsible for the estrogen induced expression of the α_{2u}-globulin gene.

Discussion.

The initial induction response of α_{2u}-globulin gene expression by estrogen and the subsequent inhibitory effect in castrated female rats (and male rats) suggests that the effect of the hormone on the expression of this gene may involve at least two different processes. The first one is the inducible response in which the estrogen activated receptor interacts with the transcription complex and enhances transcription. In the second process, which requires continued estrogen treatment, estrogen induces other transcription factors, and these factors interact with the transcription complex to suppress the expression of the α_{2u}-globulin gene.

In our study, the α_{2u}-globulin gene in mouse L-cells transfected with the estrogen receptor gene demonstrated estrogen inducibility, a response similar to that seen upon the initial treatment of castrated females. And similar to the initial response *in vivo*, this induction is inhibited by cycloheximide, which implies the involvement of other factors. However, the requirement for sequences between -226 bp to -762 bp, which include an ERE at -591 bp implicates a direct involvement of the estrogen receptor as well. The involvement of other transcription factors in the regulation of gene expression by estrogen has been strongly suggested by the cloning and characterization of several different proteins). Nuclear protein RIP140 was found to interact specifically with the activation domain of estrogen receptor in vitro in an estrogen dependent manner (Cavailles et al., 1995). This protein was also found to interact with the estrogen receptor in intact cells and modulates its function in the presence of estrogen, suggesting that RIP140 may be a mediator of transcription activation by estrogen receptor. ERAP160, a estrogen receptor-associated protein was also found to interact with the estrogen receptor in an estrogen dependent manner (Halachmi et al., 1994). The ability of estrogen receptor to activate

transcription was shown parallels with its ability to bind with ERAP160, also suggests that ERAP160 may mediate the function of estrogen receptor. It is not clear at this moment what factors are involved in the estrogen induced transcription of α_{2u}-globulin gene, but the mouse L-cell system provides us with an opportunity to determine what these factors could be and to understand the nature of their interaction with the estrogen receptor and the transcription complex.

In cell lines transfected with plasmids that contain more than 762 bp 5'-upstream sequence, the induction of the α_{2u}-globulin gene by estrogen is about 2.2 to 3.2 fold, which is lower than that in cell lines transfected with 762 bp of 5'-upstream sequence. It is not clear that the decrease of the inducibility of α_{2u}-globulin gene by estrogen in these cell lines is due to silencing sequences in the -2.7 kb to -762 bp 5'-upstream region or other reasons. Data from more detailed analysis of this region will be required to answer this question.

A pronounced synergistic effect of estrogen and dexamethasone on the α_{2u}-globulin gene transcription was observed in cells transfected with 91R91 (Fig.2-9). The synergism seems dependent on the inducibility of this gene by estrogen as indicated in Figure 2-13. Synergistic effects have been observed with glucocorticoid receptors that bind to multiple GREs (Schmid et al., 1989), or estrogen receptors that bind to multiple EREs (Martinez et al., 1989). The effects were found to be due to the cooperative binding to their HREs. The synergistic effect between these two hormones had not been observed in any system. However on clone 91, only 50 bp separate the GRE at -513 bp from the ERE at -590 bp. The close proximity of the two sites could produce cooperative binding effects leading to a synergistic response. Since the induction of α_{2u}-globulin by either hormone requires other transcription factors, another possibility is that the recruiting of the transcription factors by both receptors enhances the transcription. Further study with clone 91 should shed more light on the receptors and the regulatory factors that are responsible for the synergism.

That ERE sequence in clone 91 is active *in vivo* is suggested by the inductive effect of estrogen on the transcription of the α_{2u}-globulin RNA. But the suppressive effect of estrogen that was observed *in vivo* was not observed in the mouse L-cell system. The addition of estrogen did not suppress the dexamethasone induced transcription even in cells transfected with long 5'-upstream sequences as indicated in Figure 2-13. So it is unlikely that the absence of suppression effect is due to a defective ERE or missing 5'-upstream sequences. There has been evidence suggesting that the suppression of gene expression by estrogen receptor may involve mediators. In the regulation of the Interleukin-6 gene by estrogen (Stein and Yang 1995), the suppression by estrogen receptor is mediated by the transcription factors NF-kB and C/EBPβ. In the case of α_{2u}-globulin gene regulation by estrogen, the requirement of continued daily estrogen treatment for up to 8 days to suppress the α_{2u}-globulin expression in male rat livers indicated a requirement for other transcription factors to cause suppression. So it is likely that the administration of estrogen induces other transcription factors, and those factors are the mediators for the suppression of α_{2u}-globulin by estrogen *in vivo*. Since the receptor binding site is conserved in most of the sequenced members of α_{2u}-globulin gene family (Fig. 2-15), but the expression of the gene family is so divergent in different tissues, and the suppression by estrogen is only observed in liver, it is likely that these estrogen induced transcription factors that can mediate the suppression effect of estrogen on the expression of α_{2u}-globulin gene are tissue specific, and most likely liver specific. The presence of cell specific factors for the estrogen suppressed gene expression has been reported (Nag et al., 1990), where the estrogen mediated inhibition of a CAT gene, which was driven by the promoter sequence of *Xenopus* vitellogenin 2A gene containing an ERE, was only observed in A431 cells but not in T47D cells. In order to study the mechanism underlying the suppression of α_{2u}-globulin a system that provides the transcription factors similar to those in the rat liver will have to be found.

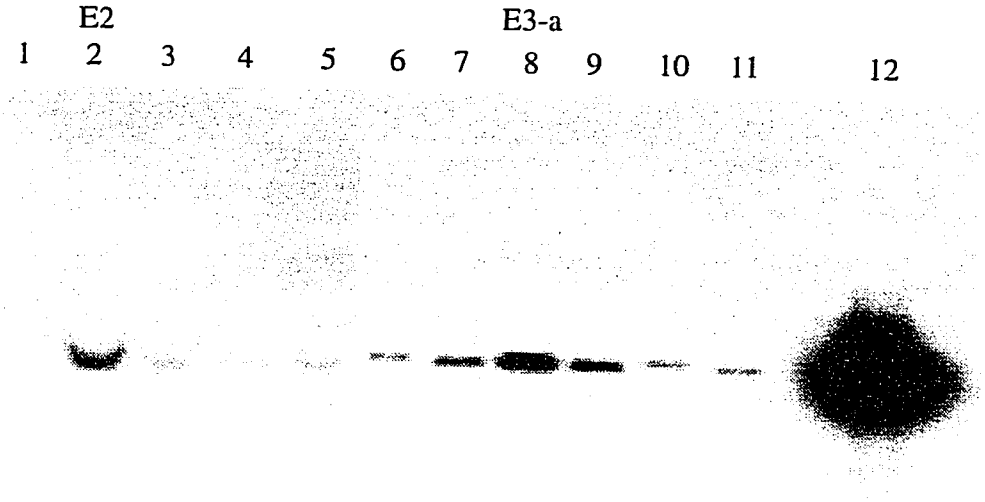


Figure 2-1. Southern blot analysis of cell lines transfected with ΔHER. 20 μg of DNA from each cell line was digested with EcoRI. The probe was a 2 kb radiolabeled EcoRI fragment from ΔHER which contains the human estrogen receptor cDNA sequence. The samples in lane 1-5 were from L-cells transfected with ΔHER. The samples in lane 6-11 were from cells transfected with 91R91 and then transfected with ΔHER. Lane 12 is plasmid DNA of ΔHER digested with EcoRI and used here as a positive control. Cell line E2 (lane 2) was used for transfections with the constructs listed in Figure 2-7. Cell line E3-a (lane 8) was used to characterize estrogen induction of α_{2u}-globulin.

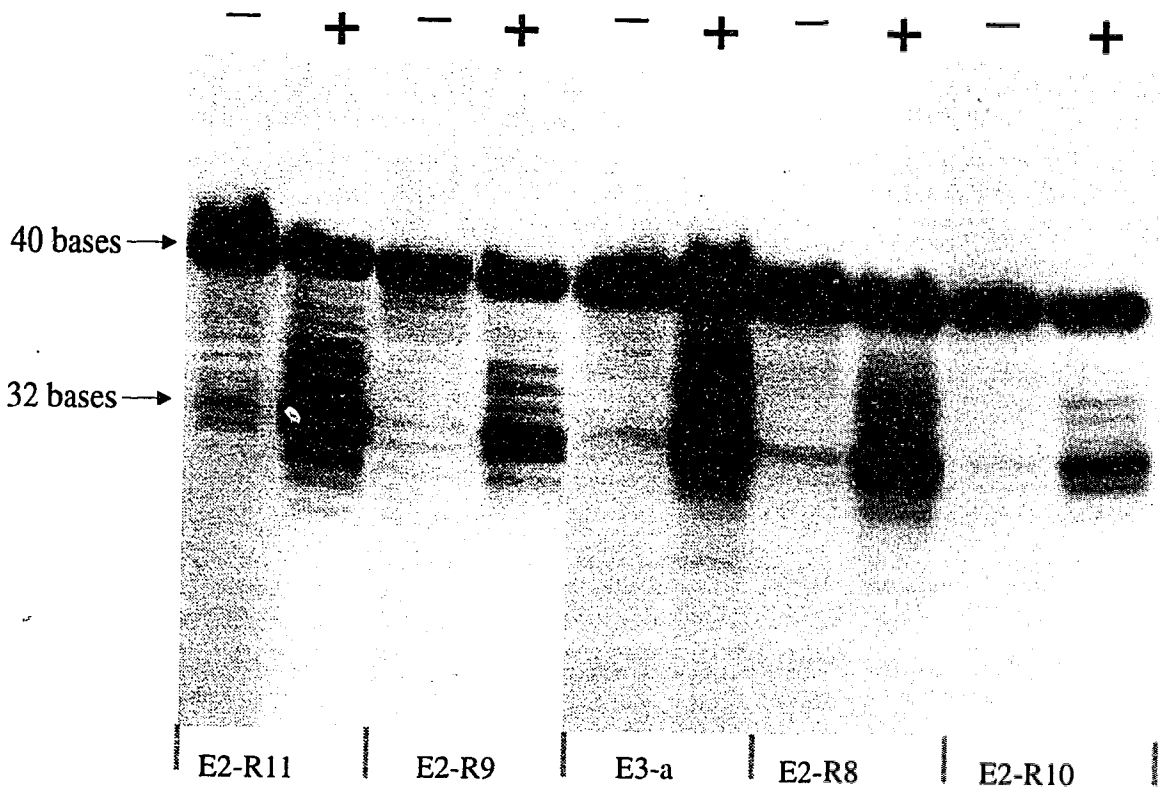


Figure 2-2. Induction of α_{2u}-globulin RNA by estrogen in mouse L-cells. Five independent cell lines were fed once every 2 days with 2.75×10^{-5}M of 17β-estrodiol (+), or without 17β-estrodiol (-). Total RNA was isolated from cells on the 9th day of induction, and 25 μg of total RNA from each sample was used in the S1 protection assay. The 32 base fragment is part of the oligonucleotide KF40 protected by α_{2u}-globulin mRNA from S1 digestion. This band was cut out to quantitate α_{2u}-globulin mRNA. The 40 base fragment that appears on the autoradiogram corresponds to the complete protection of KF40, the oligonucleotide used in the S1 protection assay, by an unknown RNA crossing the transcription initiation site of α_{2u}-globulin gene.

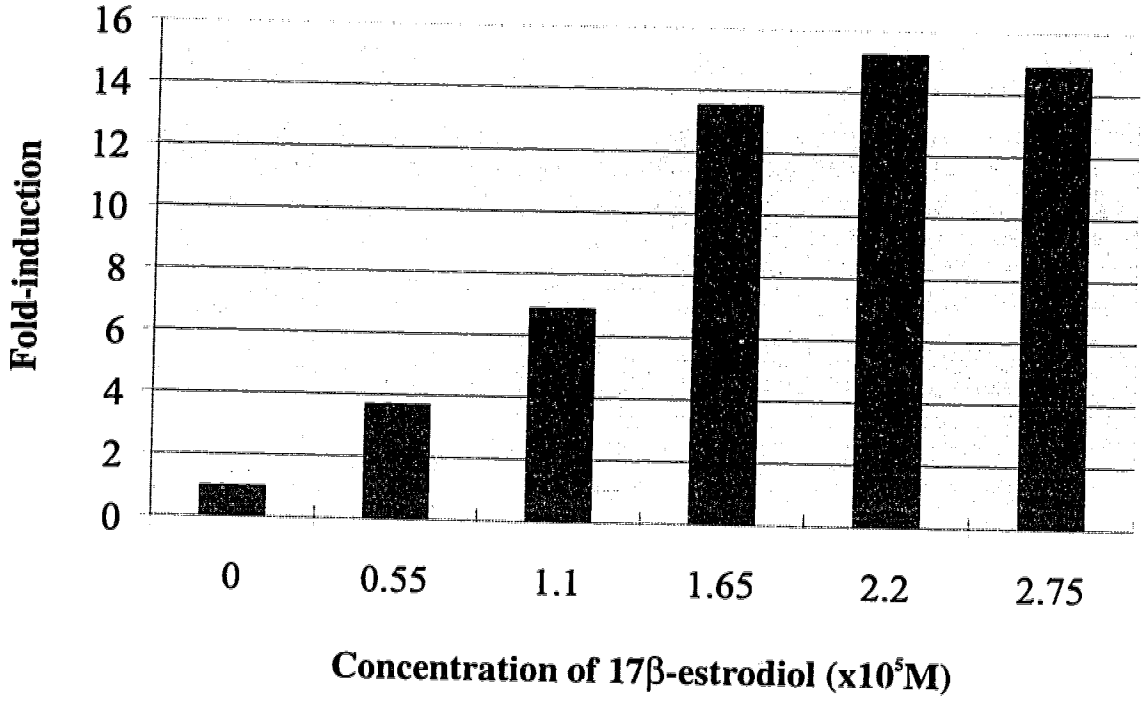


Figure 2-3. Dose-response of estrogen induced α_{2u}-globulin gene transcription. E3-a cells were treated with medium and 17β-estrodiol at the concentration indicated for 9 days. Total RNA was then isolated and the expression of α_{2u}-globulin RNA was assayed by S1 protection.

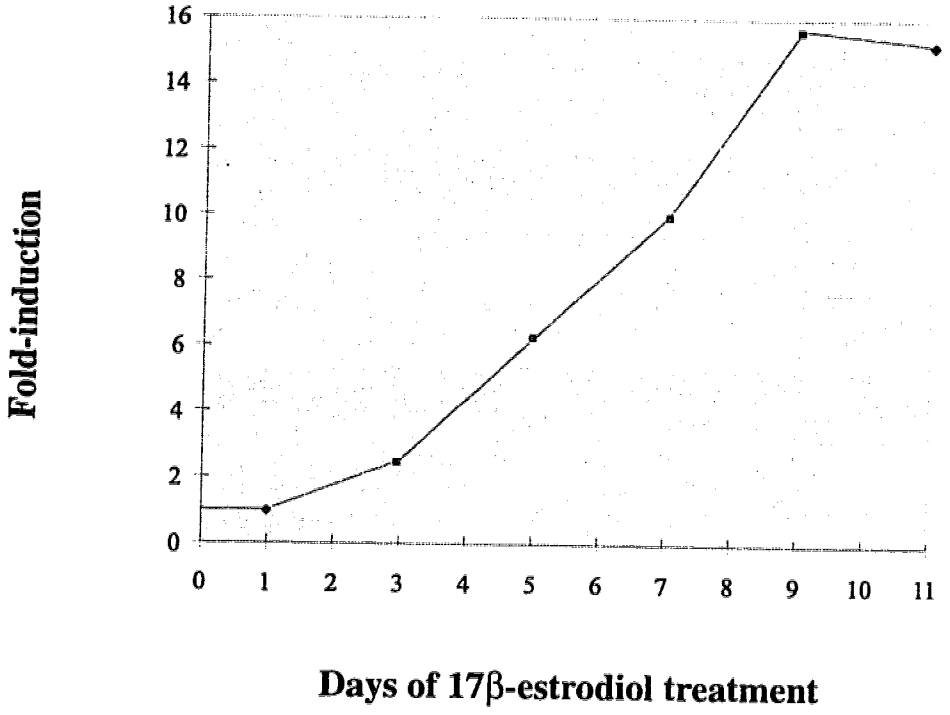


Figure 2-4. Kinetics of α_{2u}-globulin induction by estrogen. E3-a cells were fed with medium supplemented with 2.75×10^{-5}M 17β-estrodiol for 9 days. Total RNA was isolated and the transcription of α_{2u}-globulin was quantitated by the S1 protection assay.

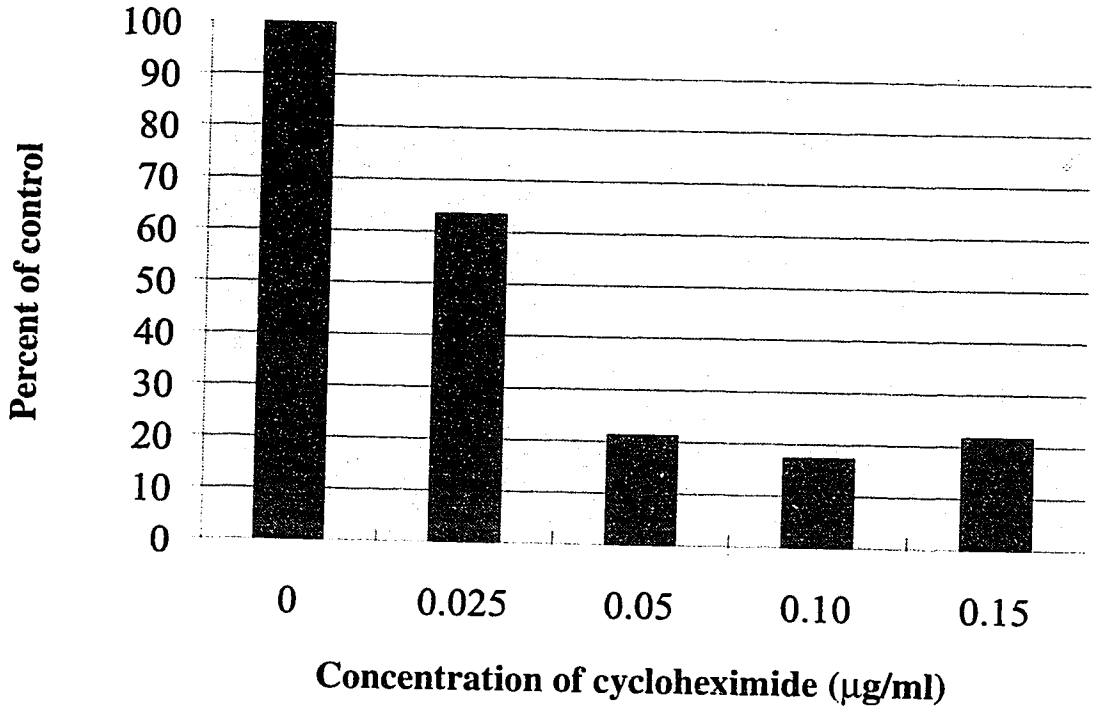
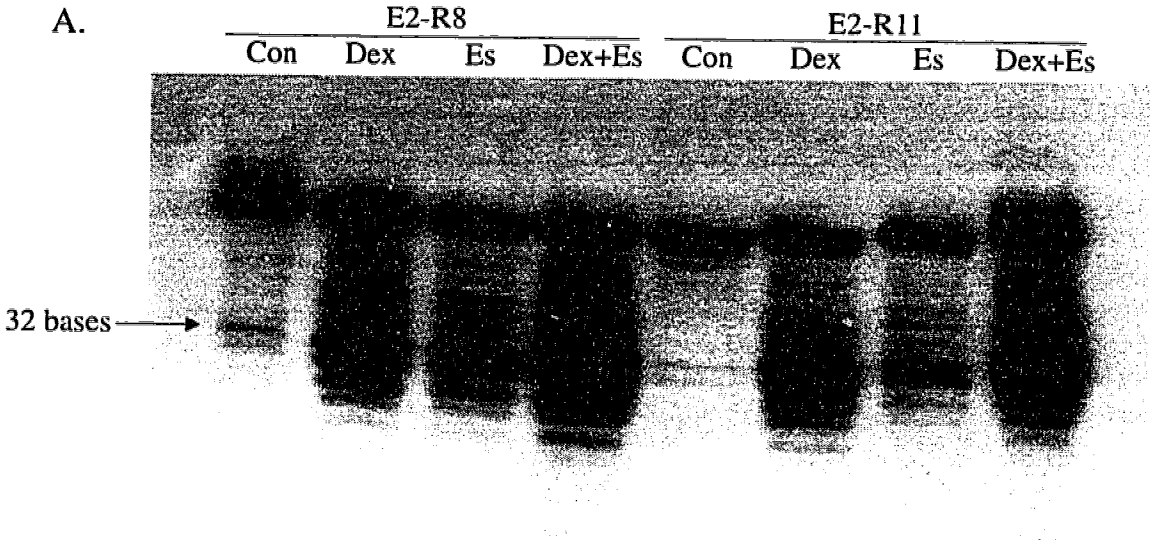


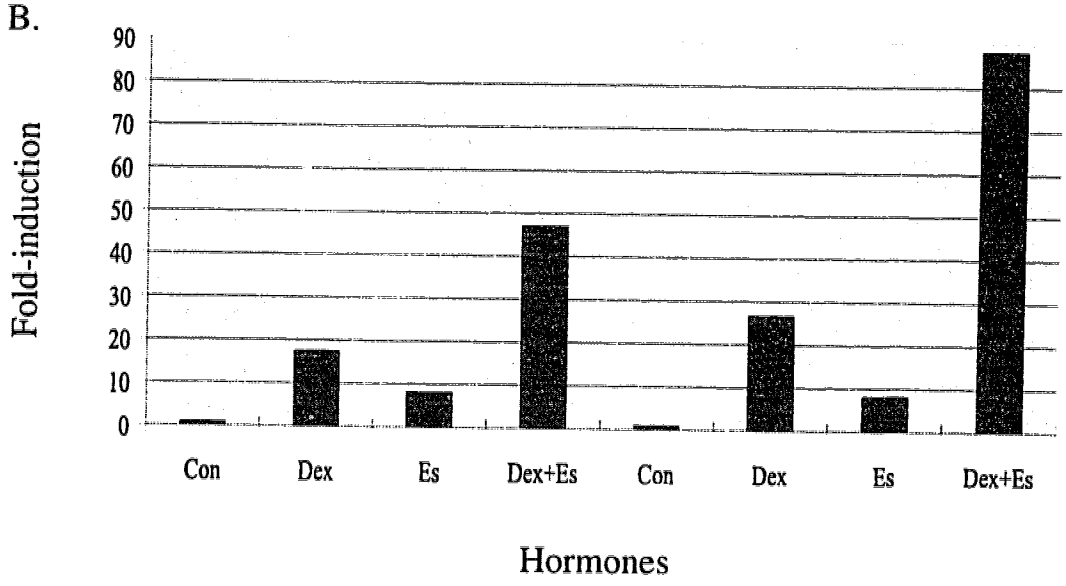
Figure 2-5. Inhibition of estrogen induced transcription of α_{2u}-globulin by cycloheximide. Cultures of E3-a cells were fed with medium containing 2.75×10^{-5}M 17β-estrodiol and the indicated concentrations of cycloheximide once every two days. After 6 days of incubation, cells were assayed for the α_{2u}-globulin RNA by S1 protection.

Figure 2-6. Synergistic effect of estrogen and glucocorticoid on the transcription of α_{2u}-globulin, clone 91. Two independent cell lines, E2-R8 and E2-R11, which are E2 transfected with 91R91, were treated with hormones as indicated above for 9 days. Total RNA was isolated, and the α_{2u}-globulin RNA was quantitated by S1 protection. A. S1 protection assay of α_{2u}-globulin gene transcripts in cells treated with hormones. B. Level of induction relative to the control (from A). Con: control. Dex: dexamethasone [$1x10^{-6}$M]. Es:17β-estrodiol [$2.75x10^{-5}$M].

A.


E2-R8
E2-R11
Con
Dex
Es
Dex+Es
Con
Dex
Es
Dex+Es
32 bases

B.


Fold-induction
90
80
70
60
50
40
30
20
10
0
Con
Dex
Es
Dex+Es
Con
Dex
Es
Dex+Es
Hormones

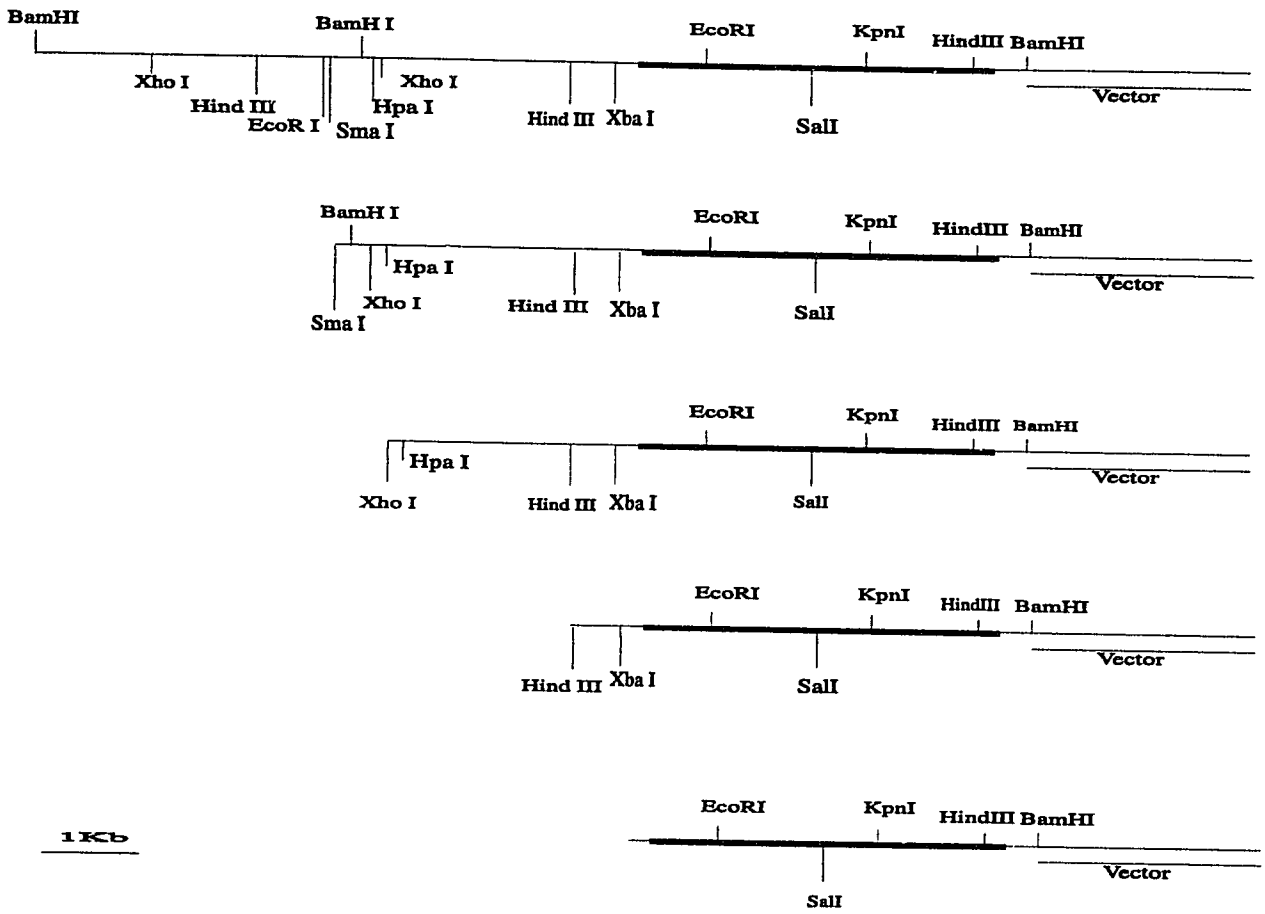

5'-upstream sequences
BamHI
BamH I
EcoRI
KpnI
HindIII
BamHI
Xho I
Hind III
EcoR I
Sma I
Hpa I
Xho I
Hind III
Xba I
SalI
Vector
91RX4
6.3 kb
BamH I
EcoRI
KpnI
HindIII
BamHI
Hpa I
Xho I
Sma I
Hind III
Xba I
SalI
Vector
91RX2
3.2 kb
EcoRI
KpnI
HindIII
BamHI
Hpa I
Xho I
Hind III
Xba I
SalI
Vector
91RX3
2.7 kb
EcoRI
KpnI
HindIII
BamHI
Hind III
Xba I
SalI
Vector
91R91
762 bp
1Kb
EcoRI
KpnI
HindIII
BamHI
SalI
Vector
91Da
226 bp

Figure 2-7. Plasmids used in the transfection of E2 cells. The construction of these plasmids is described in Chapter 1. The α_{2u}-globulin gene sequences in all plasmids are from clone 91. The vectors in all plasmids are the same vector sequence, RX, which is derived from pBR322. The bold line indicates the coding region. The 3'-downstream sequences are about 400 bp. The length of 5'-upstream sequences in each construct is indicated on the right side.

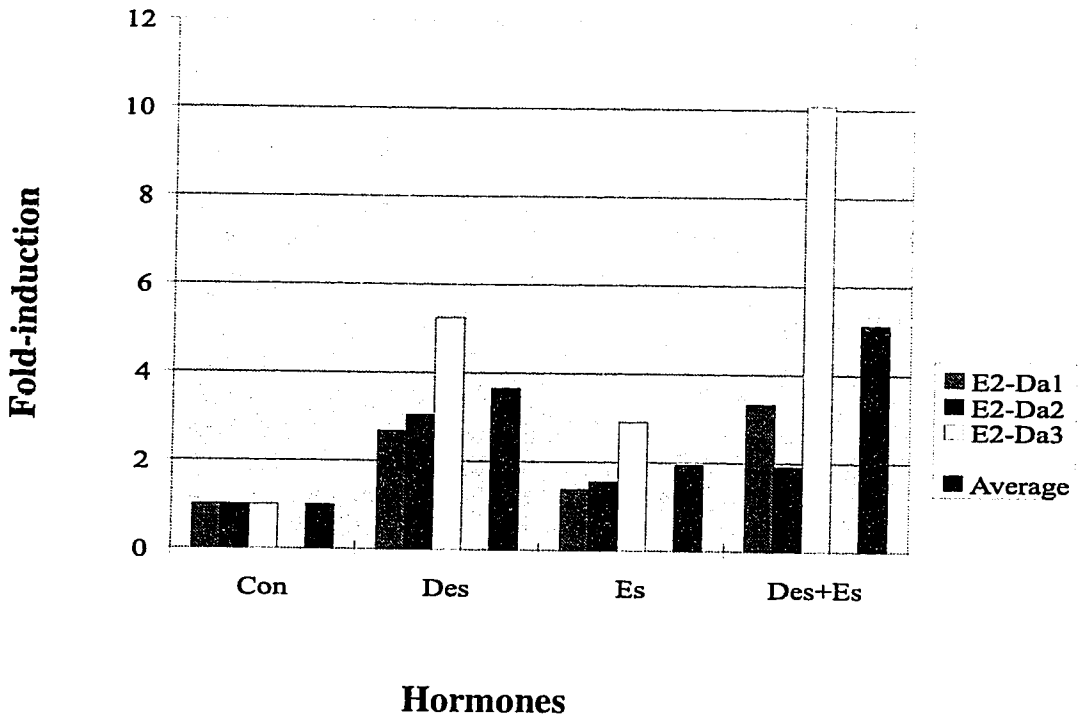


Figure 2-8. Induction of cell lines transfected with 91Da (Fig. 2-7). Three independent cell lines, E2-Da1, E2-Da2 and E2-Da3, were treated with hormones as indicated above for 9 days. Total RNA was isolated and the α_{2u}-globulin RNA was quantitated by S1 protection. Con: control. Dex: dexamethasone [$1x10^{-6}$M]. Es: 17β-estrodiol [$2.75x10^{-5}$M].

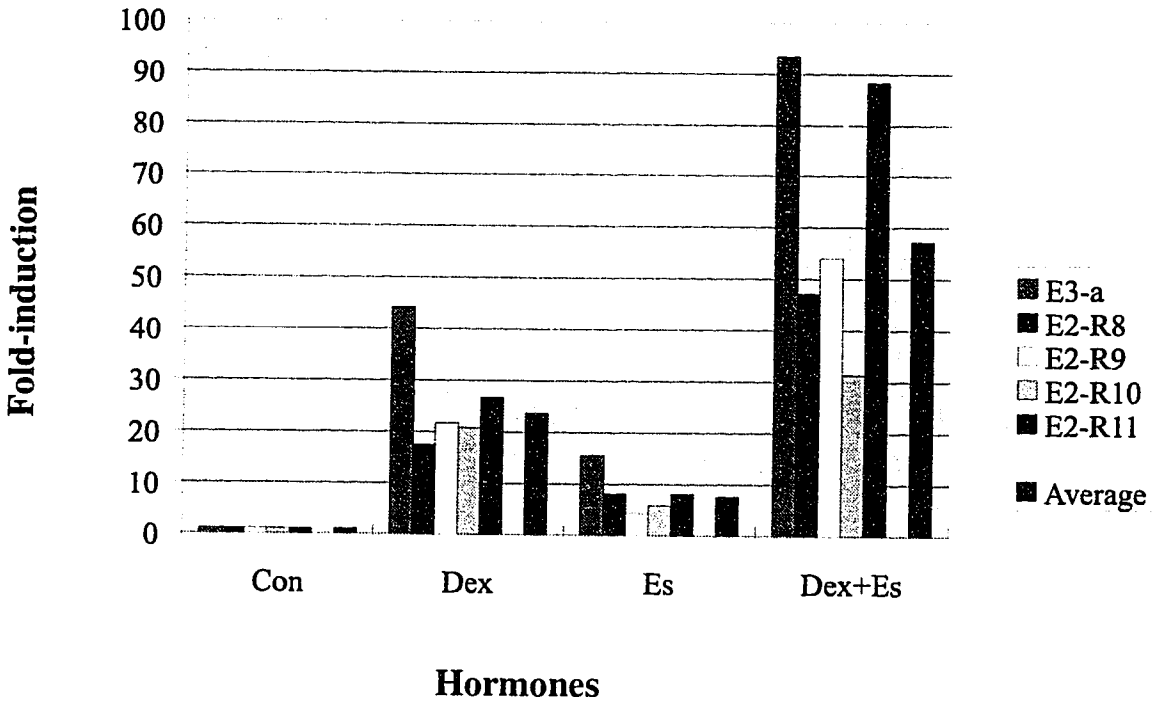


Figure 2-9. Induction of cell lines transfected with 91R91 (Fig. 2-7). Five independent cell lines listed above were treated with hormones as indicated above for 9 days. Total RNA was isolated and the α_{2u}-globulin RNA was quantitated by S1 protection. Con: control. Dex: dexamethasone [$1x10^{-6}$M]. Es: 17β-estrodiol [$2.75x10^{-5}$M].

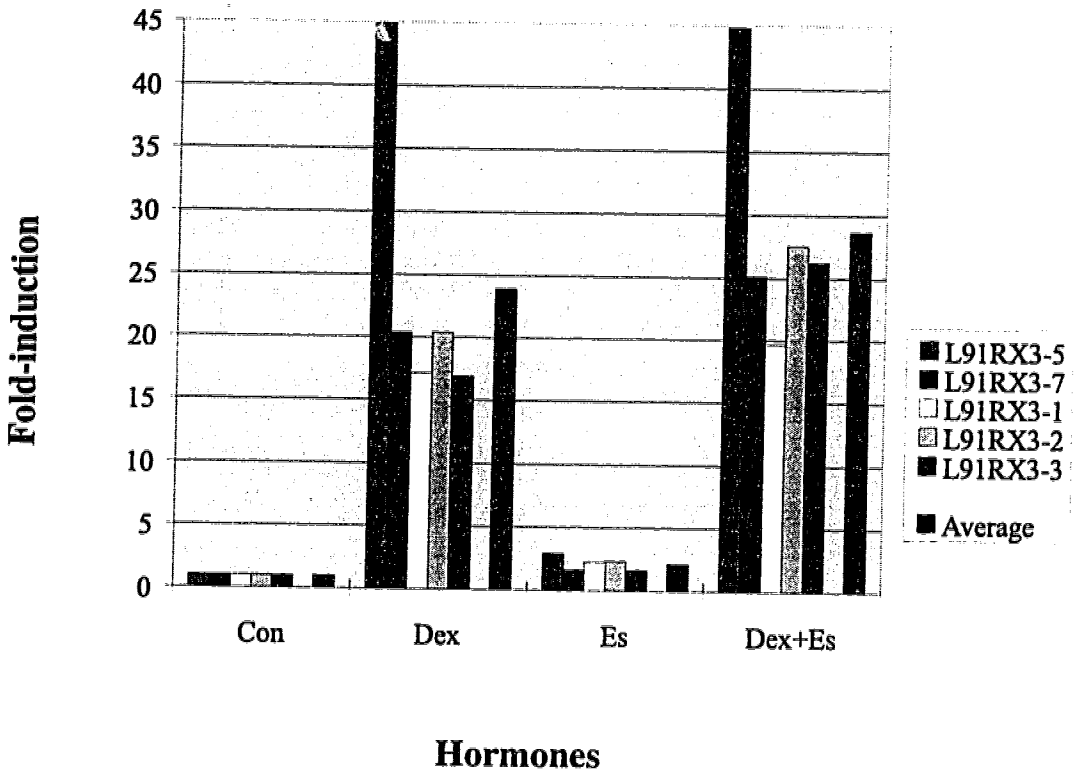


Figure 2-10. Induction of cell lines transfected with 91RX3 (Fig. 2-7). Three independent cell lines listed above were treated with hormones as indicated above for 9 days. Total RNA was isolated and the α_{2u}-globulin RNA was quantitated by S1 protection. Con: control. Dex: dexamethasone [$1x10^{-6}$M]. Es: 17β-estrodiol [$2.75x10^{-5}$M].

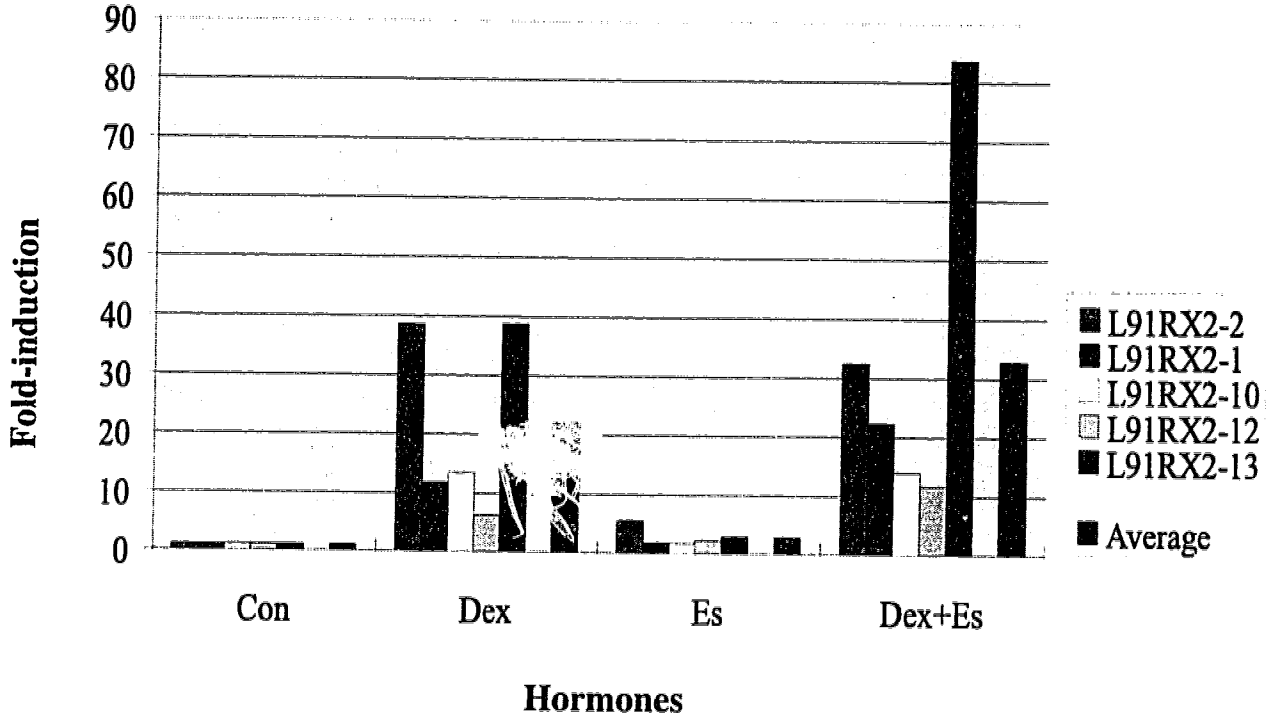


Figure 2-11. Induction of cell lines transfected with 91RX2 (Fig. 2-7). Three independent cell lines listed on the right side were treated with hormones as indicated above for 9 days. Total RNA was isolated and the α_{2u}-globulin RNA was quantitated by S1 protection. Con: control. Dex: dexamethasone [$1x10^{-6}$M]. Es: 17β-estrodiol [$2.75x10^{-5}$M].

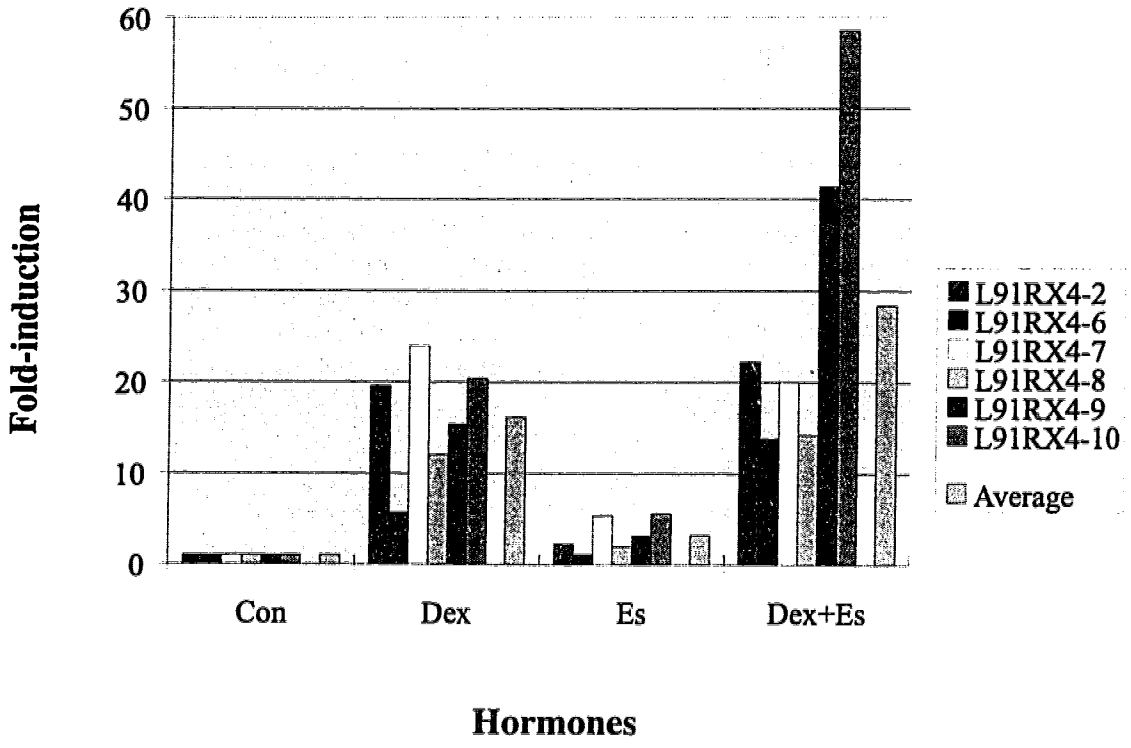


Figure 2-12. Induction of cell lines transfected with 91RX4 (Fig. 2-7). Three independent cell lines listed on the right side were treated with hormones as indicated above for 9 days. Total RNA was isolated and the α_{2u}-globulin RNA was quantitated by S1 protection. Con: control. Dex: dexamethasone [$1x10^{-6}$M]. Es: 17β-estrodiol [$2.75x10^{-5}$M].

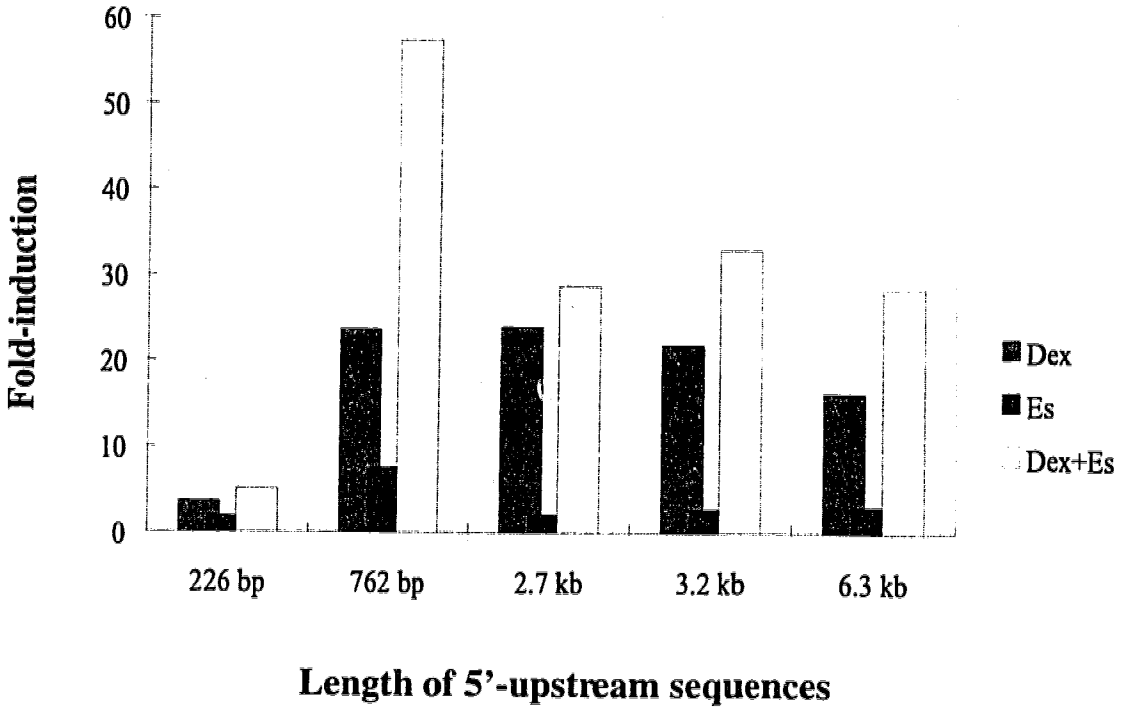


Figure 2-13. Effect of 5'-upstream sequences on the induction of the α_{2u}-globulin gene in clone 91 by hormones. The average levels of induction from Fig. 2-8 to Fig. 2-12 were plotted here. The lengths of the 5'-upstream sequences of the α_{2u}-globulin gene in the cell lines tested are indicated.

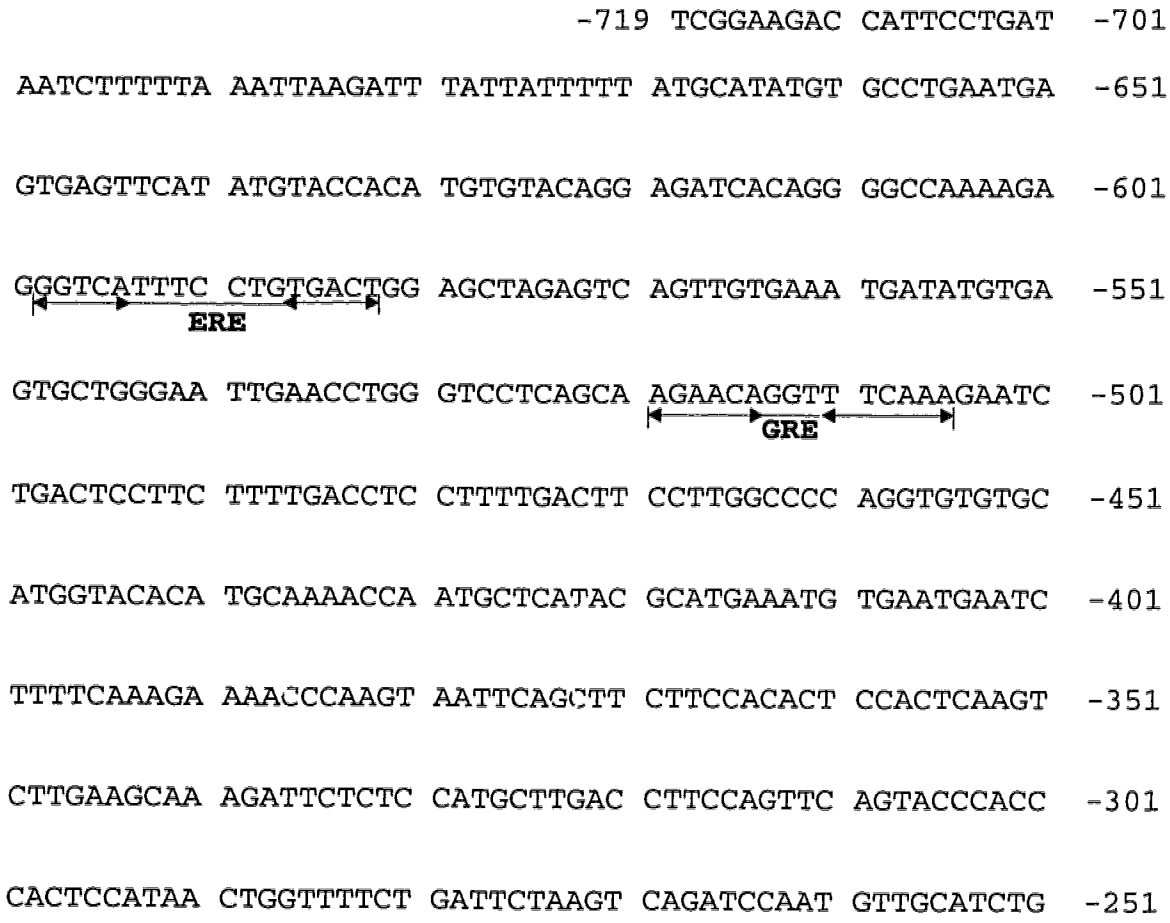


Figure 2-14. 5'-upstream sequences of clone 91. Sequences were numbered from the transcription initiation site. GRE: glucocorticoid response element. ERE: estrogen response element. The consensus sequences of ERE is GGTCANNNTGACC.

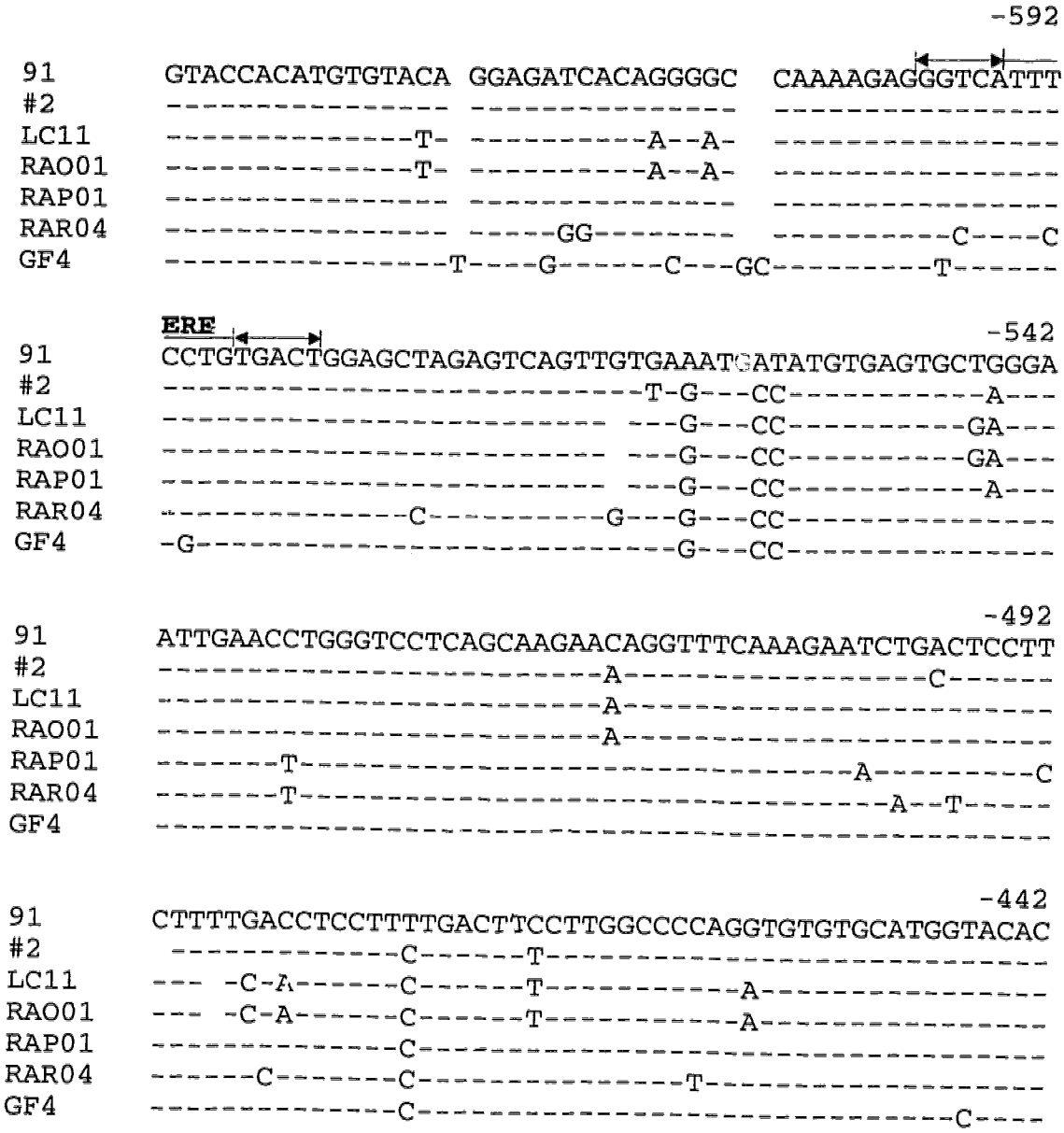


Figure 2-15. Comparison of the 5'-upstream sequences of 7 rat α_{2u}-globulin clones: 91 and #2 (see Chapter 1). LC11 (Dey and Kurtz, unpublished). RAO01, RAP01, RAR01 (Winderichx et al., 1987), and GF4 (Gao et al., unpublished). Sequences were numbered from the transcription initiation site. ERE: estrogen response element.

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Chapter 3

Differential expression of members of the α_{2u}-globulin gene family in rat tissues: characterization of α_{2u}-globulin RNA by RT-PCR and restriction analysis.

Introduction

α_{2u}-Globulin is synthesized in several rat tissues. The secreted urinary protein is synthesized mainly in the male rat liver (Roy and Neuhaus 1966; Roy et al., 1983). The expression of the α_{2u}-globulin gene in male rat livers is under developmental and multi-hormonal control. The protein appears at about 40 days of age, reaches its peak level at about 80 days and disappears at about 350 days of age. Gene expression is stimulated by androgens, glucocorticoids, growth hormone and insulin but strongly suppressed by estrogens (Roy 1979), and it is dramatically reduced by hypophysectomy, which is known to cause a multihormone deficiency. α_{2u}-Globulin is also synthesized in other tissues such as salivary, lachrymal and preputial glands of both male and female rats. The synthesis of α_{2u}-globulin in these tissues indicates a different developmental and hormonal pattern of regulation. In salivary glands, high levels of α_{2u}-globulin are detected as early as 14 days of age, but the protein is absent in adult stages (Gubits et al., 1984; MacInnes et al., 1986). Unlike its synthesis in the liver, synthesis of α_{2u}-globulin in salivary glands is independent of gender and is not affected by hypophysectomy in either sex (Laperche 1983). This indicates that the synthesis of the α_{2u}-globulin in salivary glands does not depend on hormones. In lachrymal glands, the synthesis of α_{2u}-globulin is detectable as

early as 6 days of age and is maintained into adulthood. Although the synthesis is independent of gender, it is dramatically decreased by hypophysectomy (Gubits et al., 1984). The synthesis of α_{2u}-globulin in preputial glands is also gender independent, and is not affected by hypophysectomy.

The multigene α_{2u}-globulin family consists of about 30 members (Kurtz 1981). Although the individual genes are highly similar, with 95% identity, different members of the family may contribute differently to the tissue-specific, developmental, and hormonal aspects of the expression of this gene. The α_{2u}-globulins synthesized in different tissues have similar molecular weights. But due to the slight diversity in the coding region among different members, the proteins they encode are slightly different. These isoforms of α_{2u}-globulin have different isoelectric points and therefore can be distinguished by isoelectrofocusing. Multiple isoforms of α_{2u}-globulin were detected in the liver, and in lachrymal, salivary, preputial, and mammary glands (MacInnes et al., 1986). Although many isoforms were common to more than one tissue, each tissue expressed a unique subset of isoforms. *In vitro* translation of mRNA from these tissues indicated that these isoforms of α_{2u}-globulin are the result of the expression of different subsets of α_{2u}-globulin genes (Gubits et al., 1984; MacInnes et al., 1986; Held 1985). The isoforms produced from liver mRNA did not each increase to the same extent following hormone treatment. Some isoforms are more stringent than others in their androgen requirement (Berry and Seelig 1986; Roy et al., 1987), suggesting that even in the same tissue different members of the family may be regulated differently. The differential decrease of various isoforms during aging has also been noted (Roy et al., 1983).

All this evidence suggests that the diversity of α_{2u}-globulin isoforms in the different rat tissues is largely due to differences in the regulation of individual members of the gene family. As seen from the two members of the family, clones 91 and #2, discussed in Chapter 1, different responses to dexamethasone were due to differences in the regulatory sequences. In order to correlate the studies of the regulatory sequences of the individual members with their tissue specific expression *in vivo*, it is important to be

able to distinguish among different members of the family. But the high degree of similarity between members of the family makes this very difficult. An earlier attempt to distinguish members expressed in different tissues was made by Gubits et al., (1984). By analyzing the cDNA synthesized from poly(A)$^+$ RNA from liver, salivary and lachrymal glands, it was observed that a SstI restriction site was present in salivary and lachrymal gland expressed RNA but not in liver expressed RNA. This suggests that members expressed in different tissues may be distinguishable by certain restriction enzymes. In order to identify the rat tissues in which clones 91 or #2 are expressed, a tissue specific α_{2u}-globulin RNA analysis was conducted with RT-PCR and restriction digestion to detect diagnostic polymorphisms.

Materials and Methods

Materials. Restriction enzymes, TRIzol Reagent, Taq DNA Polymerase and 10x PCR Buffer, SuperScript II RNaseH$^-$ Reverse Transcriptase and 5x First Strand Buffer were purchased from GIBCO-BRL. α-^{32}P-ATP was supplied by Amersham Corp. Guanidine thiocyanate was purchased from Sigma Chemical Co.. Tissues were obtained from 90 and 30 day old male and 90 and 25 day old female rats provided by the University Health Sciences Laboratory Animal Services.

Total RNA Isolation. Due to the small size of preputial glands, RNA from this source was isolated with TRIzol Reagent following the manufacturer's procedure. RNA from all the other tissues was isolated by a modified guanidine method (Ausubel et al., 1995). Tissues were homogenized in 7 ml guanidine solution containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol, 0.5% sarkosyl. After extraction with phenol/chloroform (1:1), samples were loaded into SW41Ti ultracentrifuge tubes (Beckman, polyallomer), which contained 2.5 ml of 5.7 M CsCl, and centrifuged at 29,000 rpm for at least 16 hours. RNA pellets were suspended in 400 µl buffer containing 5 mM EDTA, 0.5% Sarkosyl, and 5% 2-mercaptoethanol. After

extraction with 1:1 phenol/chloroform, and chloroform, RNA samples were ethanol precipitated, and then resuspended in 600 µl DEPC treated H_2O.

RT-PCR. 1 µg of total RNA from each tissue was subjected to reverse transcription. In a total volume of 10 µl, RNA was heated at 90°C for 2 min, and then chilled on ice. A mixture of 5 µl 5x First Strand Buffer, 2.5 µl 10 mM DTT, 4 µl 1.25 mM dNTP, 2 µl 20 µM oligonucleotide KAW2 (5'-CAATGATATTGTCCCTAGTG-3'), and 1.5 µl of SuperScript II Reverse Transcriptase containing 15 U of enzyme, was added into denatured RNA and incubated at 50°C for 1 hour. Samples were then heated at 95°C for 15 min, chilled on ice and frozen at -70°C for 30 min.

PCR reactions were conducted with 1/5 of the reverse transcription product in a total volume of 30 µl containing 2 µl KAW1 (5'-GATGTCTTGGAGAATTCC-3'), 2 µl KAW2, 4 µl 1.25 mM dNTP, 3 µl 10x PCR Buffer, 0.5 µl of Taq DNA polymerase containing 2.5 U of enzyme, 2.5 µl 50 mM $MgCl_2$, 2 µCi of α-^{32}P-ATP. PCR was performed under the following conditions: 1 cycle of 5 min at 95°C, 1 min at 58°C and 2 min at 72°C, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C and 2 min at 72°C. Resulting products were subjected to restriction enzyme digestion, and fractionated on 2% agarose gels. The bands were visualized by autoradiography.

Results

Transcription of α_{2u}-globulin in different tissues of rats. To maximize the detection of α_{2u}-globulin transcripts in different tissues, two oligonucleotide primers KAW1 (5'-GATGTCTTGGAGAATTCC-3') and KAW2 (5'- CAATGATATTGTCCCTAGTG-3') were synthesized based on the information of partially or completely sequenced members of the α_{2u}-globulin gene family from GenBank. KAW1 is located in exon 2 and is conserved among all 14 members that have been sequenced for this region (data partially shown in Fig 3-1). KAW2 is located in the exon 5 region, and is conserved among all 10

members that have been sequenced for this region. Figure 3-1 shows the 9 members sequenced for both the KAW1 and KAW2 regions. These two primers were used in the RT-PCR detection of the α_{2u}-globulin transcripts in different tissues of rats, and produced a 312 bp fragment. Liver, salivary gland, brain, kidney, mammary gland and lachrymal gland tissues from one 90 day old male rat, one 30 day old male rat, one 90 day old female rat and one 25 day old female rat were isolated, and the expression of α_{2u}-globulin RNA in these tissues was examined. Preputial gland RNA from the two male rats was also examined. The results (Fig. 3-2) showed that in most of the tissues where α_{2u}-globulin had been detected previously, the levels of the α_{2u}-globulin RNA were in agreement with the previous reports. α_{2u}-Globulin RNA is present at a high level in the adult male liver, at a low level in the young male liver and undetected in the liver of 25 day old female rats. It is also detected in salivary and lachrymal glands of all the animals.

Our analysis did provide some new information. For the first time, a very low level of α_{2u}-globulin transcripts was detected in the liver of the 90 day old female rat due to the sensitivity of the RT-PCR method. α_{2u}-Globulin has been observed in the kidney of male rats, but it was thought to be present as a result of its removal from circulation (Roy and Raber 1972). Our results demonstrated the presence of α_{2u}-globulin RNA in the kidneys of male rats but not female rats, indicating the α_{2u}-globulin gene is transcribed in the male rat kidneys. The expression in the kidney of the young male rat is higher than that in the kidney of the mature male rat, suggesting a gender dependent and age dependent expression of this gene in the kidneys. A low level of α_{2u}-globulin RNA is also detected in brains and mammary glands of all male and female, premature and mature rats. In the salivary glands, unlike a previous report, which suggested a decrease of α_{2u}-globulin and its mRNA as the animal matures (Gubits et al., 1984), the amount of RNA showed no visible difference among all four animals, indicating that the α_{2u}-globulin gene is constitutively transcribed. The transcription of α_{2u}-globulin genes in the lachrymal gland showed age and gender differences. It is at a higher level in male rats compared with female rats, which parallels previous reports (Held and Gallagher 1985; Gubits et al., 1984), but it is at a higher level in premature rats than that in mature rats, rather than the

opposite as reported by Gubits et al., (1984). The transcription is also at higher level in the preputial gland of the premature male than that in the mature male, indicating an age dependent expression in this tissue as well.

Identification of tissue specific restriction sites within the coding region of α_{2u}-globulin genes. Since different sets of isoforms of α_{2u}-globulin can be found in different tissues, we were interested in finding gene specific identifiers that could be used to distinguish the members expressed in different tissues. A comparison of sequences was conducted with the coding region from nine genes (partially shown in Fig. 3-1). Two (Rmgl and Rbus) are cDNAs from salivary gland mRNA. One (GF4) has not been published but is described on the Genebank entry as being constitutively expressed. The remaining six are cDNAs from liver mRNA. These clones can be classified into two groups: one includes the constitutively expressed members, GF4 and the genes expressed in salivary gland, which our data (Fig. 3-2) show are constitutively expressed. The second class of clones includes the non-constitutively expressed liver transcribed members. Although nucleotide differences at some locations seem random, there are several sites that allow us to distinguish between these two classes. Among the 36 distinguishable sites in the exon 2 to exon 6 region shown in Figure 3-1, 13 are specific to either the constitutively expressed members or the nonconstitutively expressed members. Three of these specific sites are located within the recognition sites of restriction enzymes, ApaLI, SstI, and VspI as shown in Figure 3-1, and these three enzymes were chosen to analyze α_{2u}-globulin RNA from different tissues.

Typical results from three repeated experiments are shown in Figure 3-3 to Figure 3-6. The restriction analysis of RT-PCR products from RNA of different tissues indicates that the family members expressed in these tissues can be classified into three different subsets. One subset, named A, contains the restriction sites for both ApaLI and SstI but not VspI as indicated in the salivary gland expressed members (Fig. 3-3). In fact subset A includes all the expressed genes in this tissue as revealed by the complete loss of the 312 bp RT-PCR product following digestion with either ApaLI or SstI. We infer that

subset A constitutes a fraction of the expressed genes in all other tissues as well from the observation that the intensity of the band produced by ApaLI digestion is always exactly equal to that of the SstI digestion product (Fig.3-3 to Fig.3-6). A second subset , V, contains just the restriction site of VspI. Its existence is evident, for example, in the data for the 90 day old male liver sample (Fig.3-3), where the amount of digestion with VspI exceeds that of either ApaLI or SstI. This implies there are cDNAs cut with VspI that are not cut by either ApaLi or SstI. In fact none of the VspI transcripts appear to carry ApaLi ot SstI sites as can be inferred from the triple digestion with all three enzymes. This digestion appears as the sum of that from single digestions. For example, a small proportion of the RNA from 90 day male kidney was not digested by ApaLI (or SstI) and correspondingly a small proportion was digested by VspI (Fig. 3-4). But all the RNA was cut with triple digestion, indicating that the RNA that was left undigested by ApaLI/SstI was digested by VspI and vice versa. This suggests that subset A and subset V are independent from each other. The third subset of the family members, named N, does not contain any of the restriction sites for these three enzymes as indicated, for example, by the presence of the 312 bp band left after a triple digestion of RNA from adult male liver or male preputial glands (Fig. 3-3 and Fig 3-4; lane T).

The results in Figures 3-3 to 3-6 showed that indeed different subsets of the family members are expressed in different tissues, although one subset can be expressed in many tissues. In the salivary glands of all animals, there is only one subset of members expressed, the subset A, while in the adult male liver all three subsets are expressed with subset V at a higher level than the others. All three subsets of family members are also expressed in the preputial glands of male rats, but the subset N is expressed at a higher level than the others. In some tissues that showed age and gender dependent expression of α_{2u}-globulin such as in the livers and kidneys, the expression ratio of the subsets changes during aging (Fig.3-3 and Fig. 3-4). In both tissues the expression of subset V is increased in the adult rats compared with other subsets. The expression of subset N is also dramatically increased in the liver of adult male rat. In preputial glands, in which the α_{2u}-globulin expression showed age dependence (Fig. 3-2) but not gender dependence (Murty

et al., 1987), the relative expression of different subsets varies little with age (Fig. 3-4). The expression of different subsets in the lachrymal glands, where the expression of α_{2u}-globulin gene is age and gender dependent (Fig. 3-2; Gubits et al., 1984), showed a more complex and interesting pattern. The expression of subset V, which is decreased in the adult female is increased in the adult male, whereas the expression of subset A, which is increased in the adult female is decrease in the adult male (Fig. 3-5). The expression ratio of different subsets in mammary glands seems to be the same in adult rats, but is different in young rats, where the subset A is expressed at a relatively higher level in the young male but the subset V is expressed at a higher level in the young female (Fig 3-6).

Classification of Clone 91 and #2. Total RNA was prepared from mouse L-cells transfected with clone 91 or #2. The RNA was subjected to the same analysis as tissue RNA described above. The result indicated that clone 91 belongs to subset N, which was not digested by any of these three enzymes, and clone #2 belongs to subset V, which contains a restriction site for VspI (Fig. 3-7). So based on the results of tissue RNA analysis, clone 91 could be expressed in adult male liver or preputial glands of male rats, and clone #2 could be expressed in adult male liver, adult male lachrymal gland or in lachrymal and mammary glands of young female rat. Obviously it can not be determined exactly in which tissue these clones are expressed, and it is possible that these clones are expressed in more than one tissue. But from the results it can be concluded that neither clone would be expressed in the salivary gland of rats.

Discussion

The restriction analysis of RT-PCR products from the RNA of different rat tissues indicated that indeed the members of the α_{2u}-globulin gene family are expressed differentially in different tissues. Our analysis of DNA sequences which included comparisons of α_{2u}-globulin coding region (Fig. 3-1) revealed three diagnostic restriction sites that allowed us to allocate the different genes into 3 subgroups. Additional

sequencing might reveal additional sites which would allow more subgroup or individual genes to be defined, which in turn might permit more refined tissue specific assignments of the subgroups or individual genes. At present, since subset A is the only subset expressed in the salivary gland, any genes that do not have the characteristics of subset A can not be expressed in this tissue. In other tissues, more than one subset is expressed; in the liver of the young male, the kidney of the adult male and lachrymal glands of the adult animals there are two subsets, A and V, being expressed, and in the adult male liver and male preputial glands all three subsets are expressed.

In tissues where the expression of α_{2u}-globulin is hormone dependent, the expression of all subsets is likely to be influenced by hormones. For example, in the adult male liver all three subsets are expressed with subset V being the largest fraction, whereas in the adult male kidney there are only two subsets being expressed and subset A comprises a larger proportion (Fig. 3-3 and Fig. 3-4). Nevertheless, they are all subjected to hormone regulation since they cease to be expressed in the same tissues of the adult female. But the influence of hormones on the expression of individual subsets is different. This is very apparent in lachrymal glands, for example, where the expression of α_{2u}-globulin is less affected by hypophysectomy than that in livers (Gubits et al., 1984). The expression of members in subset V is decreased in the adult female but increased in the adult male, whereas the expression of members in subset A is increased in the adult female but decreased in the adult male (see fig. 3-5). This suggests that either members in subset V are more inducible by hormones such as androgen in males or they are more sensitive to estrogen suppression in females, and vice versa for the members in subset A.

Although there is only one subset (A) of members expressed in the salivary glands, protein analysis has shown that at least five different isoforms can be identified in this tissue (MacInnes et al., 1986), indicating that there could be five different members in this subset. There are also 18 isoforms that have been identified in the preputial glands (MacInnes et al., 1986), suggesting that in each of the three subsets expressed in preputial gland, there could be a number of members. It is not possible to correlate precisely the

number of isoforms with the actual number of genes being expressed though, because post-translational modification of the protein could affect its isoelectric focusing.

It is clear that members of different subsets are regulated differently, but it is not yet clear whether members in the same subset in a particular tissue are regulated differently. In the preputial glands, where the transcription of α_{2u}-globulin is decreased during aging (Fig. 3-2), the transcription of each subset of the members seems to be decreased to the same extent (Fig. 3-4). Furthermore, the protein analysis showed that all the isoforms were also decreased to the same extent (MacInnes et al., 1986), indicating that all members in each subset were affected to the same extent during aging. In contrast, the ratio of the different isoforms expressed in salivary glands changes with age (MacInnes et al., 1986), although all of the members expressed in this tissue belong to the same subset, A. In the event that the different isoforms reflect the expression of different members of subset A, it may be inferred that the members of subset A in this tissue are not regulated in the same way. The above observations suggest that whether members of the same subset are differentially regulated may also be tissue specific.

The results of this analysis suggest that the regulation of this gene family could be more complicated than we ever thought. Different members are regulated differently in different tissues and during different stages of development. That these differences in the regulation of individual members are likely due to the differences in their regulatory sequences is suggested by the regulation of clone 91 and #2 in mouse L-cells, where clone 91 is inducible by dexamethasone, but clone #2 which has several base substitutions is not. So in order to understand how this gene family is regulated, it is crucial to understand how an individual member is regulated both *in vivo* and *in vitro*. This will require a combination of studies on the 5'-upstream sequences of a member *in vitro*, and the ability to differentiate its transcripts from transcripts of other members *in vivo*. Although it is perhaps not as extensive as it could ultimately be, the method discussed here provides a simple way to identify the possible tissues in which a cloned gene could be expressed. The challenge is to find additional diagnostic methods, with

more information from mRNA sequences, to track down the transcripts of a particular family member in tissues and to correlate the 5'-upstream sequence analysis to its expression *in vivo*.

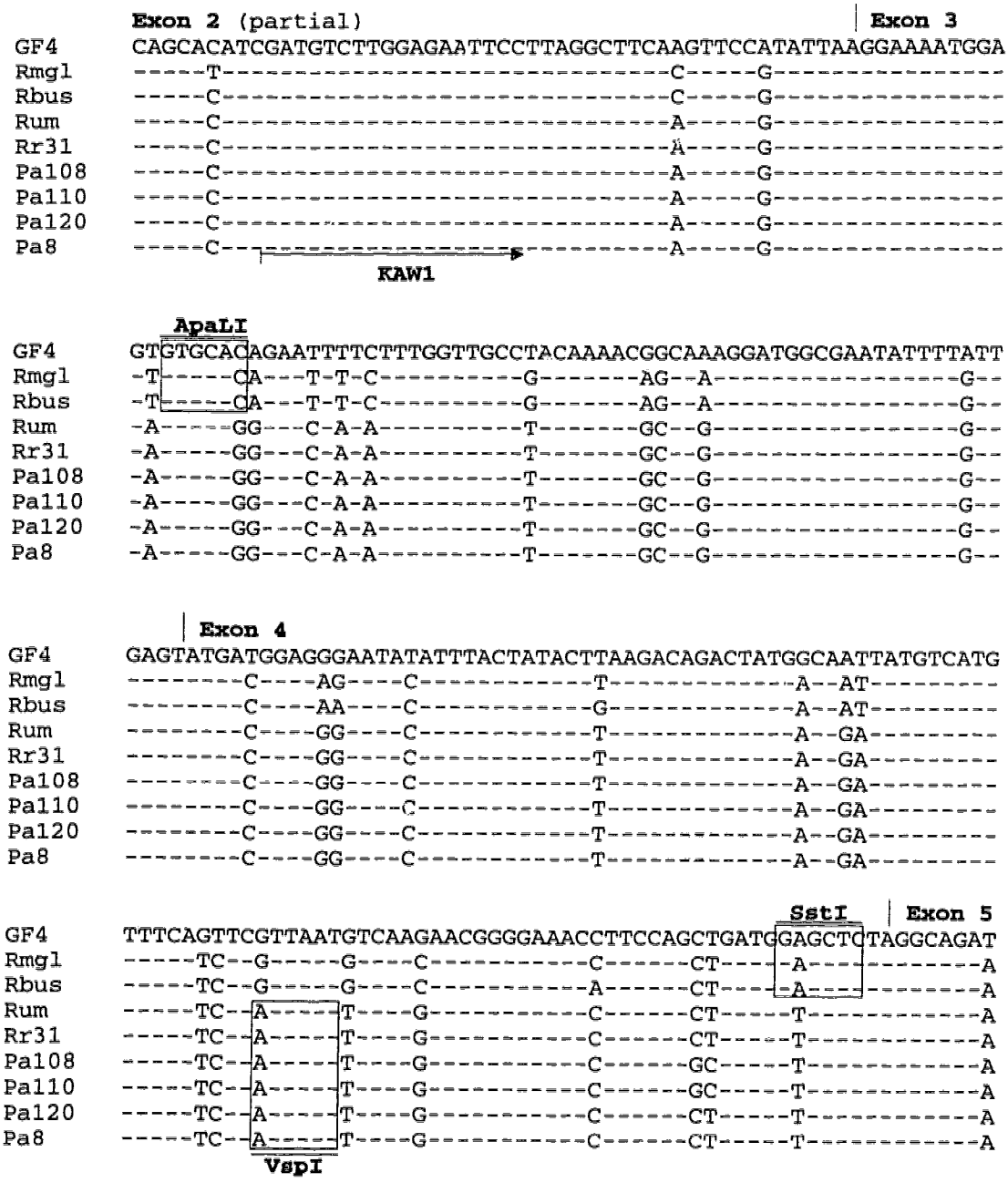

Figure 3-1. Comparison of part of the coding sequences of 9 rat α_{2u}-globulin clones. GF4 is a constitutively expressed genomic DNA clone (Gao, F., et. al. Unpublished) from which the intron sequences have been removed. Rmg1 (Gao et al., 1989) and Rbus (Laperche et al., 1983) are cDNA clones derived from rat salivary gland mRNA. Rum, Rr31, Pa108, Pa110, Pa120 and Pa8 are cDNA clones derived from rat liver mRNA (Unterman et al., 1981; Kurtz and Dey unpublished; Ichiyoshi et al., 1987).

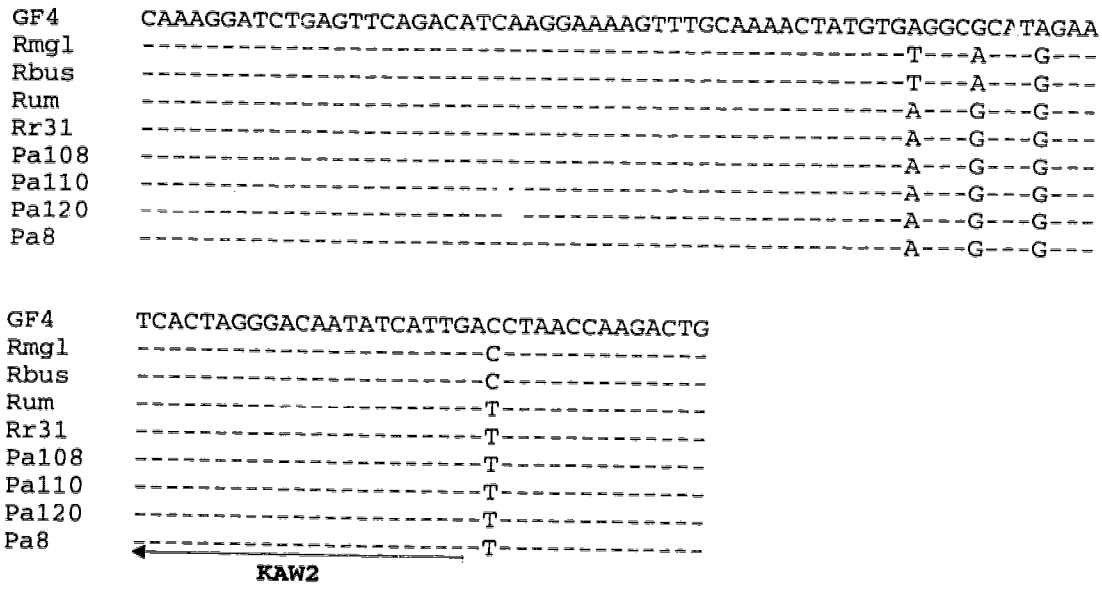

Figure 3-1. Continued.

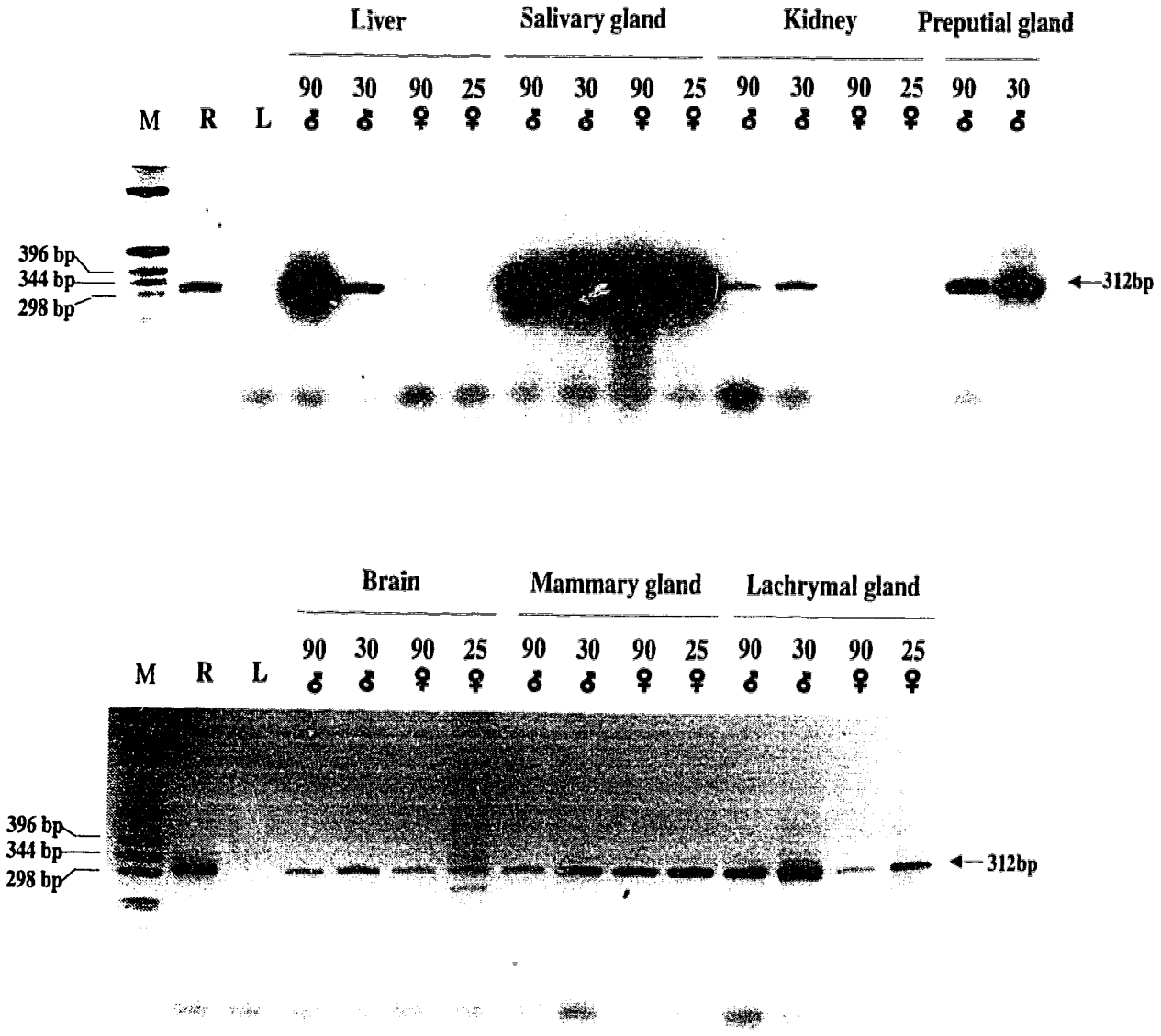

Figure 3-2. Detection of α_{2u}-globulin RNA in different tissues of rats. 0.2 μg of total RNA from the tissues indicated was used in RT-PCR using the KAW1 and KAW2 primers. Products were fractionated on 2% agarose gel. A negative image of an ethidium bromide stained gel is shown here. The result shown here is a typical result of three repeated experiments. The number above each lane indicates the age of animal (days). M: molecular weight marker. The sizes of relevant bands are indicated on the left side. R: RNA from a mouse L-cell transfected with a α_{2u}-globulin gene, clone 91, was used as a positive control. L: RNA from mouse L-cell, used as a negative control. The predicted size of RT-PCR product is indicated on the right side.

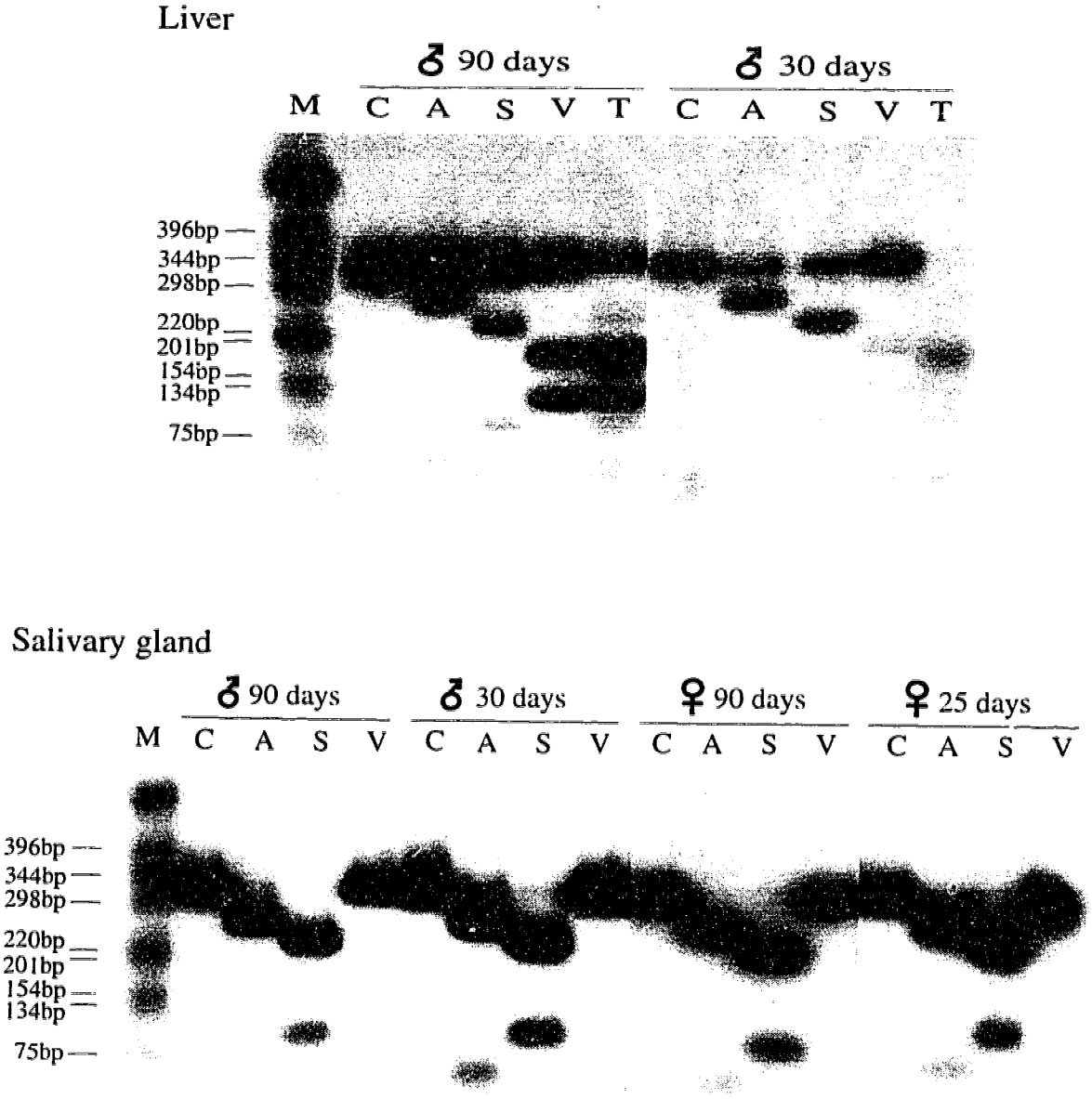

Figure 3-3. Restriction analysis of RT-PCR products from rat liver and salivary gland RNA. The gender and age of the animal are indicated above. Samples are loaded as follows: C: undigested RT-PCR product, A: ApaLI digested, S: SstI digested, V: VspI digested, and T: triple digested with all three enzymes. M: molecular weight marker, the sizes of bands are indicated on the left side.

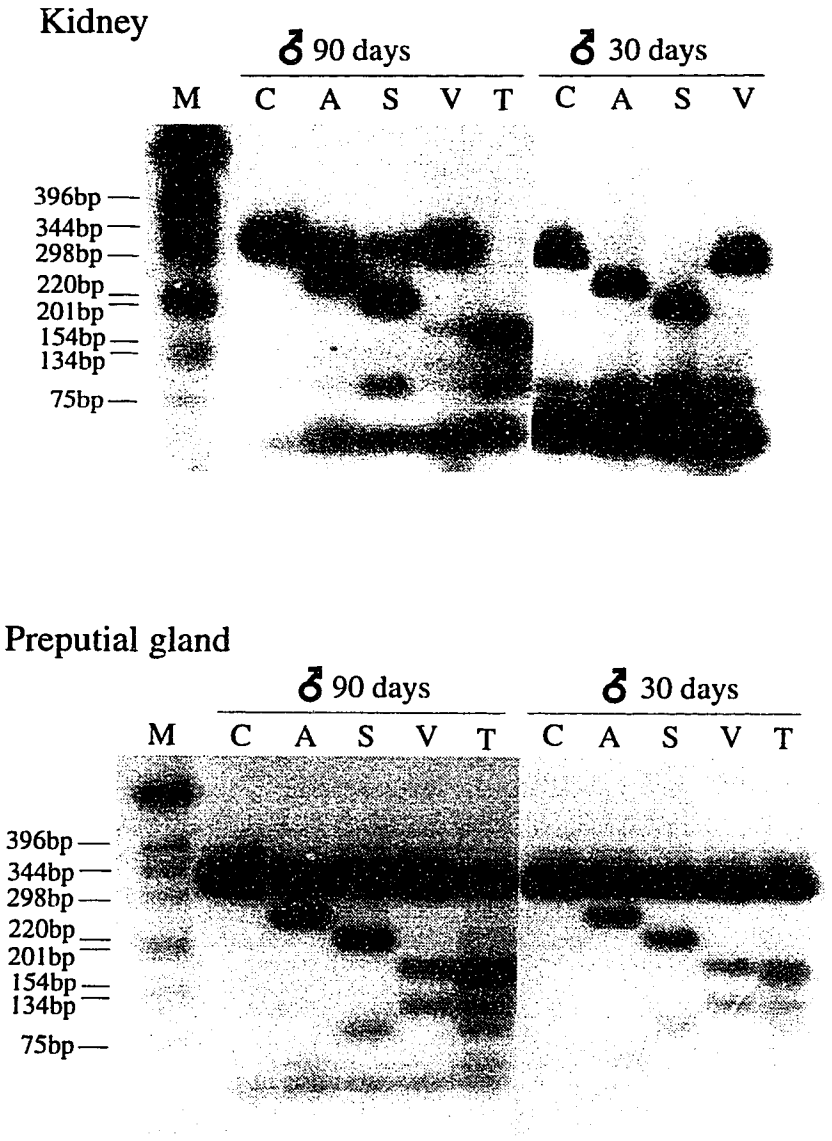

Figure 3-4. Restriction analysis of RT-PCR products from kidney and preputial gland RNA. The gender and age of the animals are indicated above. Samples are loaded as follows: C: undigested RT-PCR product, A: ApaLI digested, S: SstI digested, V: VspI digested, and T: triple digested with all three enzymes. M: molecular weight marker, the sizes of bands are indicated on the left side.

Lachrymal gland

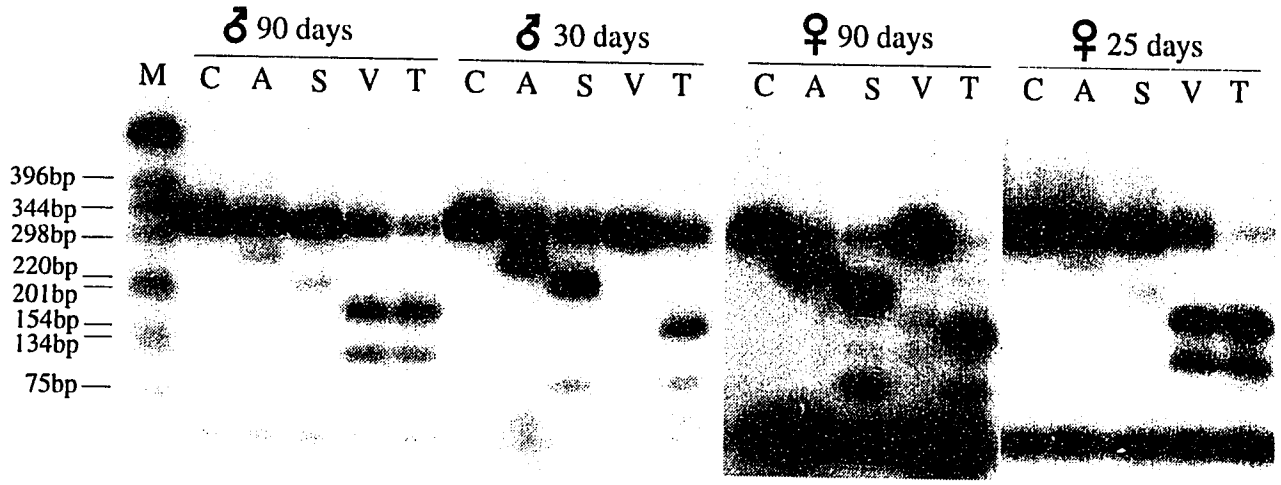

Figure 3-5. Restriction analysis of RT-PCR products from lachrymal gland RNA. The gender and age of the animals are indicated above. Samples are loaded as follows: C: undigested RT-PCR product, A: ApaLI digested, S: SstI digested, V: VspI digested, and T: triple digested with all three enzymes. M: molecular weight marker, the sizes of bands are indicated on the left side.

Mammary gland

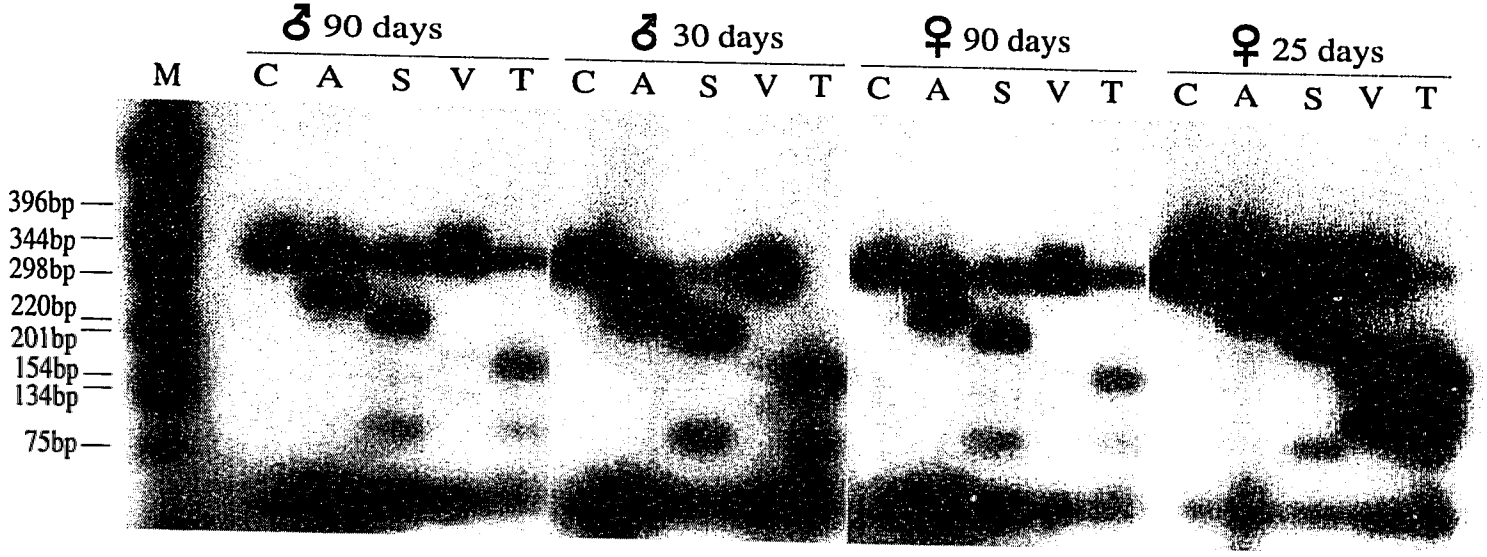

Figure 3-6. Restriction analysis of RT-PCR products from mammary gland RNA. The gender and age of the animals are indicated above. Samples are loaded as follows: C: undigested RT-PCR product, A: ApaLI digested, S: SstI digested, V: VspI digested, and T: triple digested with all three enzymes. M: molecular weight marker, the sizes of bands are indicated on the left side.

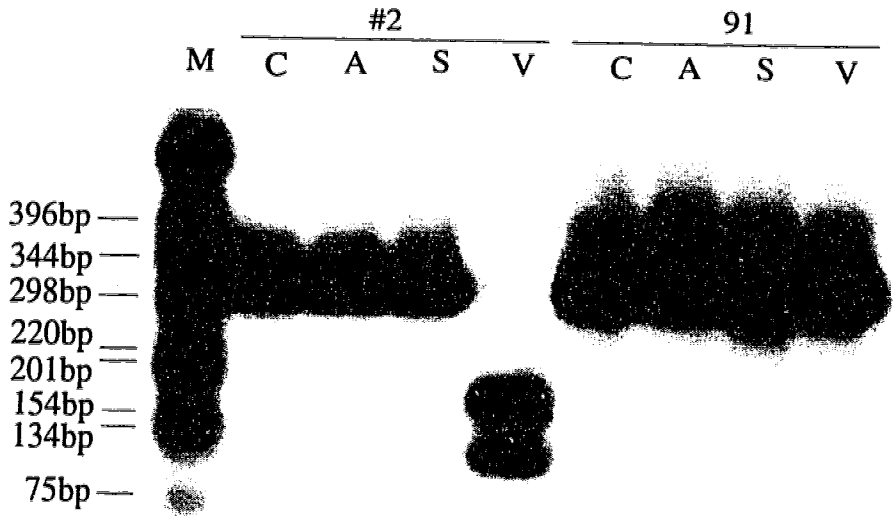

Figure 3-7. Restriction analysis of RT-PCR products from mouse L-cells transfected with clone 91 and #2. RNA from clone #2 was obtained by inducing a clone #2 transfected E2 cell line with multi-hormones. Samples are loaded as follows: C: undigested RT-PCR product, A: ApaLI digested, S: SstI digested and V: VspI digested. M: molecular weight marker, the sizes of bands are indicated on the left side.

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Future perspectives

The results in this study have revealed that the tissue specific, developmental and hormonal controlled expression of α_{2u}-globulin is due to the differentiated regulation of individual members of this large gene family. Therefore the understanding of the regulation of the expression of α_{2u}-globulin *in vivo* will rely on the understanding of how individual genes in the family are regulated. This will require knowing what are the 5'-upstream regulatory sequences involved in the regulation of each individual gene and how the differences among the regulatory sequences of family members are associated with the differences in their regulation *in vivo*. A detailed analysis of clones 91 and #2 represents an instructive way to begin this analysis since they differ in the 5'-upstream sequences, they respond differently to the dexamethasone treatment and they belong to different subgroups indicating that they could be subject to different regulation *in vivo*.

In this study with clone 91, it was shown that the 5'-upstream sequence up to -762 bp is necessary and sufficient for the dexamethasone induction and the region between -762 bp and -226 bp contains sequences specific for dexamethasone induction, since sequences up to -226 bp still maintained the ability for basal transcription. Although a GRE site has been located in the region between -762 bp and -226 bp, it is not clear whether there is any other sequence in this region important for the dexamethasone induced expression. Because protein synthesis was shown to be necessary for full induction, binding sites for transcription factors induced by dexamethasone may lie in this region. This question could be addressed by studying the *in vivo* footprints on clones 91 and #2 following dexamethasone treatment. A comparison of these footprints, from an inducible clone 91, and a noninducible clone #2, will reveal additional sites that are required for the dexamethasone induction.

Another approach to this question would be to make hybrid clones using the 5'-upstream sequences of clone 91 to rescue the inducibility of clone #2. The initial hybrid could be constructed by replacing the -762 bp to -230 bp (HindIII to XbaI) fragment of clone #2 with that of clone 91. If this hybrid clone is inducible, it would be an indication that those base substitutions between -230 bp and the transcription initiation site would not affect the inducibility of the clone #2. Further replacement with smaller fragments would reveal sequences that are important for the dexamethasone induction.

The combination of the above approaches would give us information of the important sites in the 5'-upstream sequences. A final confirmation of the functional significance of the sites could be conducted by *in vitro* mutagenesis, which could prove the necessity of these sites include GRE to the dexamethasone induction.

The results in the study with clone 91 showed that the 5'-upstream sequence between -762 bp and -226 bp is not only important for the regulation of the α_{2u}-globulin gene by dexamethasone but also important for the regulation by estrogen. Both hormones induce the expression of this gene when this region is included in the 5'-upstream sequences, and both inductions required the synthesis of transcription factors prior to the induction. It would be interesting to know whether these transcription factors are DNA binding proteins and whether the two hormone systems share the same transcription factors. This could be addressed by comparing the *in vivo* footprints produced by either hormone induction, which could reveal the presence of any additional DNA binding proteins. With regard to the identification of the non-DNA binding proteins that may be involved in these inductions, other approaches that address protein-protein interactions, such as the two hybrid system, would have to be used. This is of particular interest in light of the synergistic effect that was observed in the induction by both hormones, in which protein-protein interactions are likely to be involved.

Studies on how these two individual genes are regulated would eventually have to expand to more members in the gene family before an understanding of the complex mechanism of the regulation of α_{2u}-globulin genes *in vivo* would be possible.