THE ACTION OF THE V-ERBA ONCOGENE WITHIN THE REGULATORY
AND STRUCTURAL NETWORKS OF XENOPUS LAEVIS OOCYTES

A thesis
submitted in fulfilment
of the requirements for the degree
of
Doctor of Philosophy
in
Cellular and Molecular Biology
by
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University of Canterbury
1995
To my parents, Kurt and Lieselotte, and in memory of my grandfather

Josef Gustav Kurt Nagl, M. D. (1918, Charles University, Prague)
The universe is infinite in all directions, not only above us in the large but also below us in the small. If we start from our human scale of existence and explore the content of the universe further and further, we will finally arrive, both in the large and in the small, at misty distances where first our senses and then even our concepts fail us.

Emil Wiechert, physicist (1896)
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Abstract

Expression in *Xenopus* oocytes revealed differential modes of regulation of the constitutive *trans*-activation domains present in the thyroid hormone receptor (TR) and its oncogenic homolog v-ErbA. The nuclear environment of oocytes induced a constitutive *trans*-activation function in TR, but not v-ErbA, at hormone-inducible thyroid hormone response elements (TREs), which was further enhanced by thyroid hormone at a subset of TREs. In contrast, DNA-protein interactions at a response element which is induced by unliganded TR in mammalian cells mediated constitutive *trans*-activation by both TR and v-ErbA. These findings indicate that the responses of the ligand-independent *trans*-activation domains of TR and v-ErbA to cell-specific and TRE-mediated induction are not equivalent. Pre-injection of nuclear protein extract from anterior pituitary cells converted v-ErbA into a strong constitutive activator at a response element from the rat growth hormone gene. Thus, the dominant negative phenotype of v-ErbA can be abolished by direct or indirect interactions with tissue-specific proteins.

The growth-promoting properties of the *v-erbA* oncogene have so far exclusively been linked to dominant repression of the anti-mitogenic roles of TR and retinoic acid receptor. Ultrastructural analysis revealed that, when expressed in *Xenopus* oocytes, v-ErbA induced early to intermediate meiotic changes occurring prior to chromosome condensation. The effects of v-ErbA on cell cycle reentry in oocytes were not mimicked by a dominant negative mutant of TR, suggesting that v-ErbA did not initiate meiosis by antagonizing endogenous TR. v-ErbA-induced meiosis occurred independently of the cAMP/MPF signal pathway, but required mRNA synthesis and translation. These findings suggest that v-ErbA mediated the release from G2 arrest by activating expression of a cell cycle inducer(s).
I. Introduction

1. General Introduction

(1) Cancer as a disease of cellular signalling networks

The question of what causes cancer is central to biology, since malignancy represents disturbances in two fundamental processes of higher organisms. These two characteristics are, firstly, the ability to proliferate or make identical copies of individual cells and, secondly, the ability to differentiate, which allows cells with a shared genetic make-up to perform functions which are extremely varied and complex. Cancer can be viewed as a disease of the signalling networks through which growth factors and other mitogenic stimuli cause cells to proliferate and differentiate. Defects leading to abnormal regulation of any of the network components, encoded by proto-oncogenes and tumour suppressor genes, may result in uncontrolled cell growth and neoplasia (Weinberg, 1989a).

An oncogene is a dominantly acting gene of cellular or viral origin that can induce one or more features of neoplastic transformation when introduced into cells, either alone or in combination with another gene. Normal cellular genes that can be converted to active oncogenes by mutation are called proto-oncogenes. Activated

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Abbreviations used in this chapter: AEV, avian erythroblastosis virus; AP-1, activator protein-1; DR4, direct repeat of TRE half sites separated by 4 nucleotides; MAPK, mitogen-activated protein kinase; MPF, maturation-promoting factor; RAR, retinoic acid receptor; $T_3$, 3,5,3'-L-triiodothyronine, thyroid hormone; TR, thyroid hormone receptor; TRE, thyroid hormone response element.
proto-oncogenes are thought to provide continuous proliferation signals that enhance cell growth. For example, the oncogenic protein tyrosine kinase of Rous sarcoma virus, pp60v-src, transforms cells through unregulated phosphorylation of cellular proteins (reviewed in Hunter, 1989); while the oncogenic activity of p21ras proteins, carried by murine sarcoma viruses, correlates with abnormal signal transduction by these proteins, resulting from a reduction in their GTPase activity (reviewed in McCormick, 1989). Another class of oncogenes encodes factors involved in gene transcription, such as the v-jun and v-fos products of two murine retroviruses, whose cellular homologs make up the AP-1 transcription complex. In infected mice, v-Jun and v-Fos induce sarcomas by acting as continuously active transcription factors (reviewed in Eisenman, 1989; Hunter, 1989).

Tumour suppressor genes, on the other hand, are recessive genes that may counter the effects of oncogenes by either suppressing proliferation or inducing differentiation. For example, retinoblastoma, a cancer of embryonal retina cells, arises through the loss of both functional RB-1 alleles (Cavenee et al., 1983; Knudson, 1971); and Wilms' tumour, a cancer of embryonal kidney stem cells, is associated with the inactivation of both copies of the WT1 tumour suppressor gene (Call et al., 1990; Gessler et al., 1990; reviewed in Feinberg, 1994). Tumour suppressor genes can contribute to neoplastic transformation when they become inactivated by dominant negative mutations, leading to the expression of abnormal proteins that inhibit the functions of their normal counterparts.

In this present study, novel modes of action of the v-erbA oncogene and of its cellular progenitor, the thyroid hormone receptor (TR), were characterized. v-erbA, carried by the avian erythroblastosis virus (AEV), constitutes a prototypic example of
a dominant negative oncogene, derived from a tumour suppressor gene. v-ErbA antagonizes the action of its cellular counterpart, c-erbAα, the chicken TRα (Damm et al., 1989; Sap et al., 1989; Weinberger et al., 1986). TRα, a hormone-regulated transcription factor involved in the control of diverse cellular processes, has been classified as a tumour suppressor, because it helps regulate the differentiation of haematopoietic progenitor cells (Disela et al., 1991; Fuerstenberg et al., 1992; Gandrillon et al., 1994; Rascle et al., 1994; Schroeder et al., 1990, 1992a, 1992b; Zenke et al., 1988; 1990; reviewed in Damm, 1993). Thyroid hormone (3,5,3'-L-triiodothyronine; T3), being a relatively small, lipophilic molecule, diffuses into cells through the plasma membrane and binds to TRs in the nucleus (reviewed in Brent et al., 1991; Glass and Rosenfeld, 1991). Thus, by linking the extracellular hormonal signal directly to transcriptional processes, TR acts as an abbreviated signal transduction pathway.

(2) **Retroviral oncogenes are essential tools for contemporary oncogene research**

The RNA tumour viruses, now known as retroviruses, were among the first recognized oncogenic agents of any kind. Viruses of this type, for example the Rous sarcoma virus, were first identified as leukemogenic and sarcomagenic agents in chickens 84 years ago (Rous, 1911). In 1935, Rothe-Meyer and Engelbreth-Holme reported the identification of another infectious agent capable of inducing erythroleukemia in birds (reviewed in Privalsky, 1992). Several isolates of the "avian erythroblastosis virus" were obtained, and ultimately a stable strain, designated AEV-ES4/R, was recovered and shown to be a retrovirus. An intensive investigation of the oncogenic mechanisms employed by these viruses, however, began only in
contemporary times, made possible by the development of assays for transformation of cultured cells and biochemical methods for analysis of viral genomes and proteins. Consequently, four decades elapsed before the study of AEV-ES4/R was resumed (Graf et al., 1976; Graf and Beug, 1978), with a subsequent focus on the cooperative action of the two AEV-associated oncogenes, v-erbA and v-erbB (Graf and Beug, 1983; Frykberg et al., 1983; Vennström and Bishop, 1982).

Highly oncogenic retroviruses, including AEV, are the most rapid and efficient oncogenic agents known (reviewed in Teich et al., 1985). Viral oncogenes therefore provide a good experimental model system to study oncogenesis. Furthermore, the widespread presence of activated oncogenes in human tumours suggests that there are common mechanisms governing transformation by oncogenic retroviruses and oncogenesis by other carcinogenic agents (reviewed in Varmus, 1989). The rates of base substitutions for retroviral genes is roughly $10^6$ times higher than that for their cellular counterparts (Gojobori and Yokoyama, 1985). The high rate for the retroviral genes can be attributed to a high mutation rate caused in the process of reverse transcription (Temin, 1989), and potentially allows retroviruses to undergo multiple mutations and recombinations before they are subject to selection. These observations have given rise to the hypothesis that the evolution of oncogenic retroviruses is a mutation-driven process, whose stages are analogous to the spontaneous mutations leading to the activation of cellular proto-oncogenes (Temin, 1988; reviewed in Weinberg, 1989b). In nonretroviral cancers, genetic instability and the concomitant accumulation of mutations results in tumour cell heterogeneity. This process provides malignant tumours with great biological diversity, which enables them to succeed in a competitive environment. Consequently, cancer cells not only
develop aggressive proliferation and metastasizing potential, but also resistance to natural and synthetic drugs (reviewed in Pienta et al., 1989). The explanatory power of the "oncogene hypothesis" has provided a strong impetus to efforts aimed at understanding the precise role of mutations found in retroviral and cellular oncogenes.

(3) Contributions of v/c-erbA research to an understanding of human cancer

It was recently reported that male mice transgenic for v-erbA develop hepatocellular carcinoma, demonstrating that v-erbA can promote neoplasia in mammals (Barlow et al., 1994). Based on the fact that v-ErbA contributes to oncogenic transformation by dominantly repressing TR and the closely related retinoic acid receptor (RAR) (Schroeder et al., 1992a, 1992b), dominant negative forms of TR and RAR could reasonably be expected to be involved in human cancers. Dominant negative forms of TR, with several genetic lesions resembling those present in v-ErbA, have been identified in the rare syndrome of generalized thyroid hormone resistance (GTHR) (reviewed in Privalsky, 1992). In TRs from individuals suffering from GTHR, point mutations in the ligand binding domain result in ligand binding-deficient receptors that act as dominant negative inhibitors when coexpressed with the wild-type TR. Elevated rates of cancer development have not been observed in affected individuals, suggesting that the expression of at least these particular dominant negative TR alleles does not result in cancer in humans. However, the possibility that a link between other dominant negative forms of TR and human cancer might be found in the future cannot yet be excluded. Interestingly, a dominant negative RAR has been implicated in promyelocytic
leukemia (PML) in humans (reviewed in Damm, 1993; Privalsky, 1992). The unliganded PML-RARα fusion product, consisting of a truncated RARα gene translocated to the PML locus on chromosome 15, is thought to act through a dominant negative mechanism to interfere with promyelocytic differentiation. This indicates that inhibition of cellular differentiation by dominant negative nuclear receptors of cellular origin does indeed occur in human cancers.

An understanding of the processes which help to prevent cancer is one of the primary aims of cancer research. In this regard, perhaps the most important outcome of studies of v/c-erbA to date has been the characterization of c-erbA as a tumour suppressor gene (reviewed in Damm, 1993). Not only does TR/c-ErbA mediate differentiation of erythroid progenitor cells, but it also is involved in: (i) repression of mitogenic signalling components, such as repression of the AP-1 transcription factor complex in chicken embryo fibroblasts (Desbois et al., 1991a, 1991b; Zhang et al., 1991), and inhibition of platelet-derived growth factor B/c-sis gene expression in glial cells (Iglesias et al., 1995); (ii) inhibition of proliferation (Gandrillon et al., 1994); and (iii) induction of programmed cell death (apoptosis) (Gandrillon et al., 1994). Finally, the study of v-ErbA has been invaluable in the molecular dissection of functional domains of nuclear receptors, and has greatly contributed to our understanding of the action of dominant negative oncogenes. These aspects of v-ErbA are discussed in detail in Chapters II and III.
Oocytes of *Xenopus laevis*, the African clawed frog, can be used as an exceptionally efficient assay system to characterize the function of both cis- and trans-acting factors in gene expression (Fig. 1) (Gurdon and Melton, 1981; Heikkila, 1990; Wang *et al*., 1991). For example, *Xenopus* oocytes have recently been employed in studies of transcriptional regulation by oestrogen receptors (Theulaz *et al*., 1988; McKenzie *et al*., 1990; Watson, 1991), and by a recombinant trans-activator protein, which consisted of the potent activation domain of the herpes simplex virus virion protein VP16 and the DNA binding domain of the yeast activator GAL4 (GAL4-VP16) (Xu *et al*., 1993). Unlike expression studies in transfected cultured cells, oocyte microinjection offers the possibility to directly introduce cell components, such as nuclear proteins, as well as gene templates, and thereby allows the *in vivo* reconstitution of cell-specific nuclear environments. This advantage of the *Xenopus* oocyte expression system is illustrated by studies of β-globin promoter activation in the presence of nuclear extracts from erythroid tissues (Rungger *et al*., 1990); silencing and activation of the mouse interleukin-2 gene promoter by protein extracts from resting and mitogen-induced primary T-lymphocytes (Mouzaki *et al*., 1991); and stimulation of sea urchin early and late H2B histone gene expression by a gastrula nuclear extract (Maxson *et al*., 1988).

Oncogene involvement in the signal transduction events mediating control of the cell cycle can be studied by maturation assays carried out in *Xenopus* oocytes (reviewed in Smith, 1989). For example, the oncogenic p21\textsuperscript{cm} product (Birchmeier *et al*., 1985; Pomerance *et al*., 1992), the *v-mos* protein (Freeman *et al*., 1990), the
Figure 1. Stage V (Dumont, 1972) *Xenopus laevis* oocytes. The dark pigmented animal pole and the unpigmented vegetal hemisphere are shown. Bar: 1 mm.
pp60v-src product (Spivack et al., 1984), and the oncogenic form of neu tyrosine kinase (Narasimhan et al., 1992) have been studied in oocytes. Unlike most vertebrate somatic cells, which are arrested in G₀, Xenopus oocytes are arrested in prophase at the Gₛ/M border of meiosis. Progesterone stimulation of oocytes leads to the activation of pre-existing mitogen-activated protein kinase (MAPK) and a cyclin B/CDC2 complex, known as maturation-promoting factor (MPF), and subsequent resumption of the cell cycle (reviewed in Kosako et al., 1994a; Maller, 1990; Smith, 1989). Since expression of oncogenes, or microinjection of oncogenic proteins, can also lead to cell cycle reentry, oocytes offer a useful system in which the pathways mediating oncogene action can be analyzed.

2. Nature and Scope of this Investigation

(1) Aim 1: How do the trans-activation and repression domains of TR and v-ErbA function in Xenopus oocytes?

A central issue arising from the discovery of retroviral oncogenes is how a viral oncogene differs functionally from its normal progenitor. To address this question, the potential for differential cell-specific modes of action distinguishing TR from its retroviral homolog v-ErbA was tested in Xenopus oocytes. For the characterization of TR and v-ErbA function, regulatory networks, involving TRs, mammalian and avian thyroid hormone response elements (TREs), and cell-specific nuclear protein extracts, were reconstituted in oocytes. The findings of this study are described and discussed in Chapter II.
(2) **Aim 2: Does v-ErbA initiate cell cycle reentry in Xenopus oocytes?**

The growth-promoting properties of the v-erbA oncogene have so far exclusively been linked to dominant repression of the anti-mitogenic roles of TR and RAR (Desbois *et al.*, 1991a, 1991b; Gandrillon *et al.*, 1994; Schroeder *et al.*, 1992a, 1992b; Sharif and Privalsky, 1991; Zhang *et al.*, 1991). Chapter III describes the results of meiotic induction assays and ultrastructural studies in *Xenopus* oocytes, which showed that the v-erbA product induces early to intermediate meiotic events by activating *de novo* gene expression. The findings of this study suggest that v-ErbA does not initiate cell cycle reentry by antagonizing endogenous TR in oocytes.
II.

Constitutive Trans-activation by the Thyroid Hormone Receptor and a Novel Pattern of Activity of its Oncogenic Homolog v-ErbA in Xenopus Oocytes

1. Introduction

This chapter describes how transcriptional control by the α and β isoforms of thyroid hormone receptor (TR) is modulated by (i) the nuclear environment of Xenopus oocytes, (ii) the structure of the DNA control elements, termed thyroid hormone response elements (TREs), at which TRs are bound, and (iii) heterodimerization with the retinoid X receptor (RXR). The oncogenic counterpart of TR, v-ErbA, was tested for its ability to dominantly repress TR at a range of TREs in the context of Xenopus oocytes. v-ErbA was shown to be converted to a transcriptional activator by binding to certain TREs and by the presence of injected tissue-specific factors from anterior pituitary cells. In the introduction, the structural and functional differences between TRα, TRβ, and v-ErbA, and the mechanisms of transcriptional control by these nuclear receptors are reviewed.

Abbreviations used in this chapter: ELISA, enzyme linked immunosorbant assay; lysTRE, TRE from the chicken lysozyme gene; maITRE, TRE from the rat malic enzyme gene; rGH, rat growth hormone; rGH3TRE, TRE from the third intron of the rat growth hormone gene; RXR, retinoid X receptor; T3, 3,5,3'-L-triiodothyronine, thyroid hormone; TR, thyroid hormone receptor; TRE, thyroid hormone response element; TREp, palindromic TRE.
(1) **Evolutionary relationships and diversity of thyroid hormone receptor genes**

TRs are members of the steroid/thyroid hormone receptor gene family of sequence-specific transcription factors, inducible by their cognate ligands (Detera-Wadleigh and Fanning, 1994; Laudet et al., 1992). In addition to receptors for thyroid hormone (T3), members of this family include the receptors which mediate the effects of glucocorticoids, androgens, oestrogens, progestins, mineralocorticoids, 1,25-dihydroxyvitamin D3, all-trans retinoic acid, and 9-cis retinoic acid, as well as a number of related orphan receptors without known ligands (Detera-Wadleigh and Fanning, 1994; Laudet et al., 1992; reviewed in Beato, 1989; Evans, 1988). Nuclear hormone receptors are key regulators of metabolism, reproduction, development and tissue-specific differentiation (reviewed in Beato, 1989; Chatterjee and Tata, 1992; Evans, 1988). Recently, a nuclear receptor, named farnesoid X-activated receptor, was characterized which is activated by the intracellular metabolite farnesol, a component of the mevalonate pathway in mammalian cells (Forman et al., 1995a). Interestingly, the isolation of this receptor suggests the existence of a potentially novel metabolite-controlled intracellular signalling system in vertebrates.

TRs, like other transcription factors, are composed of modular functional domains whose action is dependent on cellular context (domains A to F; Fig. 2A) (reviewed in Glass and Rosenfeld, 1991; Green and Chambon, 1986). TRs are encoded by two distinct but closely related genes, c-erbAα and c-erbAβ, which map to chromosomes 17 and 3 respectively in humans (reviewed in Brent et al., 1991). Both genes express several alternatively spliced isoforms. The TRα genes in rats and humans express the T3-binding isoform TRα1 along with c-ErbAα2, which does not
Figure 2. Modular composition and amino acid sequence similarity of TR isoforms and v-ErbA. (A) Schematic of a generalized TR represented as a rectangle; indicated within are the A/B, C, D, E and F subregions. Below are shown the various receptor domains thought to be involved in transcriptional activation, dimerization or interaction with other proteins, DNA recognition and binding (including the P- and D-box elements), and the regions thought to mediate nuclear localization (adapted from Privalsky, 1992). (B) Comparison of primary structures of human TRβ1 (hTR beta1), rat TRα1 (rTR alpha1) and v-ErbA, the proteins used in this study. v-erbA is expressed as a fusion protein made up of the retroviral gag protein and the v-erbA product. Numbers within regions corresponding to the C and E domains represent percent amino acid identity with hTRβ1 (adapted from Glass and Rosenfeld, 1991). Little or no significant homology is observed when comparing other regions of the receptors.
A DNA-binding Hormone-binding

N A/B C D E F C

Transcriptional activation
Dimerization/Protein-protein interactions
DNA recognition
Nuclear localization

B

<table>
<thead>
<tr>
<th>A/B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
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<tr>
<td>100</td>
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</table>

hTR beta1

|     | 90 | 87 |

rTR alpha1

| gag | 88. | 86 |

v-ErbA
bind T₃ because of alternative splicing at the C terminus (reviewed in Brent et al., 1991; Glass and Rosenfeld, 1991). The closely related chicken α gene expresses only chicken TRα which is over 90% identical at the amino acid level to rat TRα1 (rTRα1) and human TRα1 (hTRα1) (Brent et al., 1991). The TRβ gene expresses TRβ1 and TRβ2 that differ only in their N-terminal A/B regions, which are distinct from the A/B region of TRα1 (Brent et al., 1991; Glass and Rosenfeld, 1991). rTRα1 and hTRβ1, employed in this study, are over 85% identical at the amino acid level in their DNA binding and ligand binding domains (Fig. 2B).

(2) The role of thyroid hormone receptors in the regulation of eukaryotic gene transcription by RNA polymerase II

Cells regulate the transcription of virtually all their genes; for example, in order to establish a differentiated phenotype during development, or in response to extracellular signals, such as T₃. Transcription of eukaryotic protein-encoding genes by RNA polymerase II involves the assembly of multiprotein complexes on distal control DNA elements, called enhancers, and proximal promoters (reviewed in Maldonado and Reinberg, 1995). The highly regulated nature of transcription complex assembly provides a finely tuned mechanism for controlling gene expression. Transcription is mediated by two distinct classes of transcription factors. The first class comprises general transcription factors which are necessary for accurate initiation of transcription. These factors include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIG/J, and TFIIF (reviewed in Maldonado and Reinberg, 1995; Buratowski and Sharp, 1992). TFIID is a multiprotein complex consisting of the TATA box-binding protein (TBP) complexed with a number of TBP-associated
factors (TAFs) (Maldonado and Reinberg, 1995). The binding of TFIID is thought to be the first step in transcriptional initiation, which is followed by the stepwise recruitment of the additional transcription factors and RNA polymerase II, and ultimately results in the initiation of transcription (Pugh and Tjian, 1992).

The magnitude of transcriptional activation is greatly affected by the second class of sequence-specific transcription factors, transcriptional activators and repressors, which act to either promote or inhibit the formation of an active transcriptional initiation complex (reviewed in Johnson, 1995; Ptashne, 1988; Ptashne and Gann, 1990; Tjian and Maniatis, 1994). Transcriptional activators bind to enhancers, and thereby select genes to be activated, and orchestrate the assembly of the general transcription "machinery" at the start site of mRNA synthesis by RNA polymerase II (Fig. 3) (Maldonado and Reinberg, 1995; Tjian and Maniatis, 1994). Enhancer elements, which include DNA binding sites for TR, can be located at great distances from the start site of transcription, either upstream, downstream or within a transcribed gene (Banerji et al., 1981; Moreau et al., 1981). Transcription factors bound to enhancers activate transcription by forming direct protein-protein contacts with proximal promoter factors through DNA looping (Dunn et al., 1984; reviewed in Serfling et al., 1985).

Recent in vitro transcription studies suggest that the binding of TFIIB may be the rate-limiting step for the initiation of transcription, and that several transcriptional activators act to recruit or stabilize the interaction of TFIIB within the initiation complex (Hori et al., 1995; Maldonado and Reinberg, 1995; Ptashne and Gann, 1990). Protein-protein interactions with the general transcription factor TFIIB have been implicated both in transcriptional repression by unliganded TR and
transcriptional activation in response to ligand (Fig. 3) (Fondell et al., 1993; Baniahmad et al., 1993). A short sequence in the N-terminal A/B region of DNA-bound TRα is required for efficient binding to TFIIB and acts to recruit and/or stabilize the binding of TFIIB to the transcription complex (Hadzic et al., 1995). For transcriptional repression, however, inhibitory interactions with TFIIB are not sufficient; the recently identified corepressor N-CoR is also required (Baniahmad et al., 1992; Hörlein et al., 1995).

A central question in understanding transcriptional regulation is how a relatively small number of different transcription factors can be used to achieve the high level of specificity required to regulate the complex patterns of gene expression in higher eukaryotes. At least part of the answer is that transcription factors and enhancers are composed of modular components (reviewed in Beato, 1989; Dynan, 1989; Chatterjee and Tata, 1992; Tjian and Maniatis, 1994; Gronemeyer and Moras, 1995). As exemplified by TR (Fig. 2A), a typical transcription factor contains a specific DNA-binding domain, a multimerization domain that allows the formation of homodimers or heterodimers, and a transcriptional trans-activation domain. Similarly, enhancers contain distinct sets of transcription factor-binding sites, and variations in the arrangement of the binding sites provide the potential to create unique DNA-protein complexes by forming heterodimers within and among families of transcription factors (reviewed in Karin et al., 1993; Mangelsdorf and Evans, 1992). Synergistic interactions between the proteins within the complex result in specificity, a potential for multiple regulatory controls, and a high level of transcription. Importantly, cell-specific cofactors modulate the effects of transcriptional activators and repressors in a highly tissue-specific manner.
Figure 3. Schematic of a multistep model of trans-activation by TR. The multistep model of trans-activation is derived mainly from in vitro studies of mammalian systems (adapted from Maldonado and Reinberg, 1995). Multiple cooperative interactions (symbolized by arrows) between TR, RXR, other activators (A), coactivators (CA), corepressors (CR), accessory factors (AF), and general transcription factors (TFIIA-H, TBP, TAFs), mediate activation of transcription by RNA polymerase II (RNAPII) at the initiation start site (start) of TR-regulated genes. The multiprotein complex made up of TATA-binding protein (TBP) and TBP-associated factors (TAFs) constitutes the general transcription factor TFIID. In mammalian cells, TR, associated with CR, represses transcription by inhibitory interactions with TFIIB in the absence of T3 (-T3). T3 induces both the dissociation of CR and the recruitment of CA (+T3). As a result, the TR/CA complex activates transcription by enhancing binding of TFIIB to the general transcription complex (refer to text for further details).
modular enhancer

A J ~RXR +T ~T3 ~@B ~1

promoter

start
These mechanisms are well illustrated by the multi-level control of the rat growth hormone (rGH) gene, which offers an interesting system for studying the interactions between factors that mediate cell-type specificity and the factors that mediate hormone responsiveness. The promoter of the rGH gene, which is exclusively expressed in the somatotrophic cells of the anterior pituitary gland (reviewed in Frawley, 1989), contains DNA binding sites for TR (Ye et al., 1988; Flug et al., 1987; Norman et al., 1989; Brent et al., 1989) and for the general transcription factors Sp1 (Schaufele et al., 1990a) and GHF-3 (Schaufele et al., 1990b), as well as two sites for the pituitary-specific factor Pit-1/GHF-1, belonging to the homeobox POU domain-containing family of transcription factors (Bodner et al., 1988; Lefevre et al., 1987; Schaufele et al., 1990c; West et al., 1987). An additional binding site for TR, which was employed in this present study, is located in the third intron of the rGH gene (Sap et al., 1990). TR and Pit-1 act synergistically in the activation of the rGH promoter (Schaufele et al., 1992; Ye et al., 1988), which helps to explain how the widely distributed TR can activate the expression of certain genes only in selected cell types.

(3) Specific recognition of thyroid hormone response elements by thyroid hormone receptors

The DNA binding domain of TR possesses two zinc finger motifs (Freedman et al., 1988; Luisi et al., 1991). Adjacent to each zinc finger is an amphipathic α-helix, involved in the folding of the zinc finger domains into a compact globular structure (Hard et al., 1990a and 1990b; Luisi et al., 1991; Remerowski et al., 1991; Schwabe et al., 1990). The P-box of the first α-helix, mediating TRE recognition, contains
three discriminating amino acids thought to interact directly with specific bases of the consensus motifs within TREs (Danielsen et al., 1989; Luisi et al., 1991; Mader et al., 1989; Umesono et al., 1989). Further dissection of the P-box revealed that the amino acids act independently at the level of DNA binding (Nelson et al., 1993). The D-box, adjacent to the second α-helix, provides phosphate contacts to the DNA backbone and participates in receptor-receptor dimerization (Danielsen et al., 1989; Lee et al., 1993; Luisi et al., 1991; Umesono and Evans, 1989; Umesono et al., 1991; Zechel et al., 1994a, 1994b).

TRs control expression of T3-responsive genes at cts-acting TREs (reviewed in Chatterjee and Tata, 1992). TREs are made up of six base pair consensus sequences, termed half sites, consisting of the canonical 5'-PuGGTCA recognition motif. These half-sites can be oriented as imperfect inverted, direct or divergent repeats, separated by different sized nucleotide gaps (reviewed in Gronemeyer and Moras, 1995; Privalsky, 1992). Naturally occurring promoters often show mixtures of half sites in differing orientations (Chatterjee and Tata, 1992). Specific recognition of a TRE by the receptor appears to require the appropriate orientation and spacing of the half-sites, as well as the correct sequence within each half-site (Baniahmad et al., 1990; Desai-Yajnik and Samuels, 1993; Forman et al., 1992; Glass et al., 1988; Lazar et al., 1991; Näär et al., 1991; Umesono et al., 1991). TR homodimers specifically recognize a palindromic arrangement of half-site motifs with no spacer nucleotide (reviewed in Gronemeyer and Moras, 1995), as exemplified by a synthetic response element, termed TREp (Damm et al., 1989) used in this study (Fig. 4). This requirement for a distinct number of spacer nucleotides results from the characteristics of the D-box within the DNA binding domain, which represents a
Figure 4. Thyroid hormone response elements employed in this study. Nucleotide sequences of the TRE from the rat malic enzyme gene (maTRE) (Petty et al., 1990), the TRE from the chicken lysozyme gene (Baniahmad et al., 1990), the TRE from the third intron of the rat growth hormone gene (rGH3TRE) (Sap et al., 1990), a synthetic palindromic TRE (TREp) (Damm et al., 1989), and the clone 122 TREs (Bigler and Eisenman, 1994) are shown. The orientation of individual half sites is indicated by arrows. Intervening nucleotides between half-sites are indicated by numbers. A putative half site within rGH3TRE is depicted by a broken arrow.
TRE:

GGGTTA 4 AGGACA
direct repeat
rat
maltic enzyme
type:
gen:

CCCCAG 2 TGAGGT
inverted palindrome
chicken
lysozyme

AGGTAA 6 AGTCCC 6 AGGTCA
tripartite
rat
growth hormone
(third intron)

AGGTCA 0 TGACCT
palindrome
synthetic construct

TRE1:
TAAGCCCCCAGCCCCCGACAT
CCAGGACGGCCCAAA

TRE2:
ATACCTATTACCTCATCATGTGAATAG
complex
"clone 122"
gene unknown

TRE3:
TCCGAGTGACCTCCTCGTCATTTGTTG

weak DNA-induced dimerization interface (Luisi et al., 1991; Schwabe et al., 1990; Umesono and Evans, 1989). TRs can bind TREs as monomers and homodimers (Forman et al., 1992; Ikeda et al., 1994; Lazar et al., 1991; Katz and Koenig, 1993). However, heterodimerization with mammalian auxiliary factors, most notably the nuclear receptors for 9-cis retinoic acid (RXRs), results in increased affinity for the response element in vitro and enhanced activation of gene expression in vivo (Bugge et al., 1992; Burnside et al., 1990; Heyman et al., 1992; Kliewer et al., 1992; Murray and Towle, 1989; Yu et al., 1991; Zhang et al., 1992; for a review, see Mangelsdorf and Evans, 1992).

Many naturally occurring TREs are composed of two directly repeated half-sites separated by four nucleotides (DR4), such as the TRE from the rat malic enzyme gene (malTRE) (Petty et al., 1990) employed in this study (Fig. 4). Heterodimerization of TR with RXR at DR4, which is due to a strong interaction between the dimerization surfaces located in the ligand binding domains and weaker interaction between the dimerization surfaces in the D-boxes of TR and RXR, results in cooperative DNA binding and specific recognition of DR4 TREs (Kliewer et al., 1992; Lee et al., 1993; Leid et al., 1992a, 1992b; Mader et al., 1993; Marks et al., 1992; Perlmann et al., 1993; Yu et al., 1991; Zechel et al., 1994a, 1994b; Zhang et al., 1992). Recently, the understanding of the stereochemical principles underlying specific recognition of DR4 TREs by TR-RXR heterodimers was greatly enhanced by the crystallographic characterization of the heterodimer bound to a DR4 TRE (Rastinejad et al., 1995).

In addition to palindromic and DR4 TREs, inverted palindromes which are bound by homodimers and heterodimers (Gronemeyer and Moras, 1995), like the TRE from
the chicken lysozyme gene (lysTRE) (Baniahmad et al., 1990) used in this study (Fig. 4), are found in naturally occurring enhancer elements. Finally, complex TREs, consisting of more than two half-sites arranged in differing orientations, have also been described. Two complex TREs, the TRE from the third intron of the rat growth hormone gene (rGH₃TRE) (Sap et al., 1990), and the clone 122 TREs of an as yet unidentified T₃-responsive gene (Bigler and Eisenman, 1994), were employed in this investigation (Fig. 4). At present, it remains unknown whether TR binds to complex TREs in monomeric, dimeric, or multimeric form.

(4) **Constitutive and ligand-induced transcriptional regulation by thyroid hormone receptors**

Induction of gene transcription by TR is achieved through autonomous trans-activation domains (reviewed in Gronemeyer, 1992): a constitutive trans-activation domain located in the N-terminal region of the receptor (Hadzic et al., 1995; Hollenberg et al., 1995) and, in the C-terminus, three ligand-inducible trans-activation domains, which are all required for maximum transcriptional activation (Baniahmad et al., 1995). Although transcriptional activation by TR is primarily ligand-dependent in mammalian cells, ligand-independent activation has also been observed (Bigler and Eisenman, 1994; Forman et al., 1988; Forman and Samuels, 1990; Saatcioglu et al., 1993a). In the yeast, *Saccharomyces cerevisiae*, TR functions as a ligand-independent activator with enhanced expression in the presence of T₃ (Privalsky et al., 1990). In mammalian cells, unliganded TRs, thought to be constitutively bound to hormone response elements, can suppress the basal activity of promoters containing TREs (Baniahmad et al., 1990, 1992; Damm et al., 1989; Fondell et al.,
The repressor function has been mapped to the hinge domain of TR, and was shown to be separable from its activator function (Damm and Evans, 1993). A C-terminal silencer domain has also been characterized (Banahmad et al., 1992, 1995). At present, it is not well understood how the trans-activation and repression domains are regulated in the context of the full-length receptor.

At positively regulated TREs, addition of T₃ results in the activation of transcription, suggesting that the hormone triggers a conformational change in TR that induces the activator function (Allan et al., 1992; Bhat et al., 1993; Leng et al., 1993; Toney et al., 1993). This conformational switch causes both the dissociation of corepressor proteins as well as the recruitment of coactivator(s) (Chen and Evans, 1995; Hörlein et al., 1995; Kurokawa et al., 1995; Lee et al., 1995a, 1995b). At negatively regulated TREs, TRs act as transcriptional repressors when bound to T₃ (reviewed in Chatterjee and Tata, 1992; Glass and Rosenfeld, 1991). In addition, two novel TREs were recently identified. Both the clone 122 TREs (Bigler and Eisenman, 1994) and a TRE in the Rous sarcoma virus long terminal repeat (LTR) (Saatcioglu et al., 1993a), mediate strong activation by unliganded TR, while addition of T₃ reverses this response.

(5) v-ErbA: Origins and mode of action

The v-erbA gene of the avian erythroblastosis virus (AEV) constitutes a virally transduced, highly mutated form of the chicken TRα gene, and is expressed as a fusion product with gag, the retroviral structural protein gene (for a review, see Privalsky, 1992). AEV belongs to a small group of avian replication-defective
leukemia viruses and carries two distinct oncogenes, v-erbA and v-erbB, which together induce erythroblastosis in young chicks (Graf et al., 1976; Graf and Beug, 1983). This collaborative action of v-erbA and v-erbB is discussed in detail in Chapter III.

The cooperative mode of v-erbA function reinforces the notion that the development of a transformed phenotype is most readily achieved through the actions of at least two oncogenes, the hypothesis known as "the multistep causation of carcinogenesis" (reviewed in Weinberg, 1989b). Since the v-erbA oncogene is not itself capable of overt transformation (Gandrillon et al., 1987), but cooperates with a wide variety of other oncogenes to enhance transformation (Kahn et al., 1986), it provokes two intriguing questions: What is its cooperative role, and how does it exert its subtle effects on growth and differentiation?

To date, the growth-inducing properties of v-erbA have been shown to involve interference with the function of nuclear hormone receptors, belonging to signalling pathways distinct from those induced by the mitogenic kinases. v-ErbA acts as a dominant repressor in mammalian and avian cells, blocking activation of gene expression by two differentiation-inducing factors, liganded TR and retinoic acid receptor (RAR) (Damm et al., 1989; Sap et al., 1989; Sharif and Privalsky, 1991). The v-erbA protein has sustained small N- and C-terminal deletions and 13 internal amino acid changes relative to the TR progenitor (Fig. 5) (for a review, see Privalsky, 1992). Mutations within the C-terminus of the v-erbA protein appear primarily responsible for its conversion into a constitutive transcriptional repressor, as these mutations result in loss of the ability to bind thyroid hormone at high affinity (Damm et al., 1989; Sap et al., 1989; Zenke et al., 1990; for a review, see Privalsky,
Figure 5. Comparison of the chicken TRα1 (cTR alpha1) and v-ErbA. The cTRα1 protein is displayed schematically on top, and the v-erbA protein is presented for comparison below. Arrows indicate the N- and C-terminal deletions relative to cTRα1, and the dashes indicate the locations of the 13 internal amino acid differences between cTRα1 and v-ErbA. In addition, the N-terminal fusion to retroviral gag sequences in v-ErbA is shown (adapted from Privalsky, 1992).
A/B  C  D  E  F  

cTR alpha1

v-ErbA

gag
v-ErbA has retained some binding ability, since, surprisingly, v-ErbA acts as a hormone-regulated activator in *S. cerevisiae* (Privalsky *et al.*, 1990; Sharif and Privalsky, 1991). The ability of v-ErbA to respond to hormone in yeast suggests that its hormone binding ability can be influenced by the environment. Consequently, cellular factors modulating hormone-receptor interactions may be important in the oncogenicity of v-erbA.

Several mechanisms have been suggested to account for transcriptional repression by v-ErbA, such as direct promoter silencing (Baniahmad *et al.*, 1990, 1992), competition with TR and RAR for DNA binding sites (Damm *et al.*, 1989; Selmi and Samuels, 1991), or formation of inactive heterodimers between v-ErbA and TR or RAR (Desbois *et al.*, 1991a; Schroeder *et al.*, 1992a, 1992b; for reviews, see Yen and Chin, 1994; Herskowitz, 1987). Of these mechanisms, direct promoter silencing is the least understood (reviewed in Levine and Manley, 1989; Renkawitz, 1990). That v-ErbA and unliganded TR are able to repress transcription through direct promoter silencing is shown by the fact that repression is effective on a minimal promoter (Baniahmad *et al.*, 1990, Fondell *et al.*, 1993). Moreover, both factors inhibit transcription independently of the position and orientation of the TRE to which they are bound (Baniahmad *et al.*, 1990). The following observations suggest that competition for DNA binding is critical for dominant negative activity. Firstly, mutation of the DNA binding domain of v-ErbA abrogates its dominant negative activity (Damm *et al.*, 1989). Secondly, v-ErbA homodimers, v-ErbA/RXR heterodimers and v-ErbA/TR heterodimers can form on TRES (Hermann *et al.*, 1993; Yen *et al.*, 1994). It has been proposed that one or all of these complexes may compete with normal TR complexes for binding at TRES and thereby block T3-
mediated gene activation (Yen and Chin, 1994). Finally, these different mechanisms do not have to be mutually exclusive. For instance, inactive v-ErbA/TR dimers might form on a TRE and silence the promoter by blocking the assembly of a functional pre-initiation complex (Baniahmad et al., 1990, 1992; Fondell et al., 1993; Yen et al., 1994; Yen and Chin, 1994). By default, they would also prevent TR complexes from binding to that TRE (Yen and Chin, 1994).

In addition to mutations in the hormone binding domain (Fig. 5), the v-erbA protein has acquired a substitution of one of the three discriminating amino acids within the P-box, and a second mutation in the D-box of the DNA binding domain (Fig. 6) (Bonde et al., 1991; Sap et al., 1986; for a review, see Privalsky, 1992). While the ability of the DNA binding domain of v-ErbA to bind to certain TRE sequences has been conserved (Bonde et al., 1991; Damm et al., 1989; Sap et al., 1989; Verneuil and Metzger, 1990), the two zinc finger domain mutations, and mutations in an N-terminal region outside of the DNA binding domain, alter the range of DNA target sequences the v-erbA protein can bind to relative to TR (Chen et al., 1993; Sharif and Privalsky, 1991; Smit-McBride and Privalsky, 1993; Subauste and Koenig, 1995; Zenke et al., 1990). To summarize, multiple mutations and two deletions confer an oncogenic phenotype on the v-erbA product. v-ErbA has evolved into a dominant repressor of TR, its cellular progenitor, and also exhibits an altered range of DNA binding ability relative to TR.
Figure 6. Schematic representation of the DNA binding domain of the \textit{v-erbA} protein. The two zinc finger modules are shown, using the standard one letter amino acid code. The relationship of the conserved cysteines (C) to the coordinated zinc atoms (Zn), involved in stabilization and folding of the two zinc finger modules, is emphasized. The amino acids comprising the $P$- and $D$-boxes are indicated by asterisks. The two amino acid substitutions sustained by \textit{v-ErbA} relative to \textit{cTR\alpha1} are boxed (adapted from Privalsky, 1992).
Summary of results

In this chapter, I show that *Xenopus* oocytes constitute a viable model system for the reconstitution of complex regulatory networks involving TR and v-ErbA. The nuclear environment of oocytes revealed novel functions of the constitutive trans-activation domains present in TR and v-ErbA. The results of a detailed analysis of the constitutive and ligand-enhanced trans-activation functions of both α and β isoforms of TR, exhibited at a range of TREs in oocytes, are presented. Furthermore, a constitutive trans-activator function of v-ErbA was shown to be induced by binding to a complex response element, clone 122 TREs, and by the presence of nuclear protein extract from anterior pituitary cells at rGH₃ TRE. With the exception of some additional data, the results described in this chapter have been published and a reprint of the publication is included in Appendix 1.
2. Materials and Methods

(1) Plasmids

RS-rTRα, RS-v-erbA, and tk-TREP-CAT were gifts from R. Evans (Salk Institute for Biological Studies, La Jolla, CA). The expression vectors contain rat TRα cDNA or the gag-v-erbA oncogene under the transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) (Damm et al., 1989; Thompson and Evans, 1989). tk-TREP-CAT contains a synthetic palindromic TRE linked to a herpes simplex virus thymidine kinase-chloramphenicol acetyltransferase fusion gene (tk-CAT) (Damm et al., 1989). T3-responsive CAT reporter constructs were gifts from P. J. Romaniuk (University of Victoria, Victoria, BC, Canada) and contain TREs from the promoter regions of the rat malic enzyme gene (malTRE), the chicken lysozyme gene (lysTRE), the third intron of the rat growth hormone gene (rGH3TRE), and the synthetic palindromic TRE (TREP) linked to a mouse mammary tumor virus (AMTV) LTR-CAT fusion gene (AMTV-CAT) (Nelson et al., 1993). The pTPT-CAT reporter construct was a gift from R. N. Eisenman (Fred Hutchinson Cancer Research Center, Seattle, WA) and contains a 450 bp Sau3A-BamHI fragment from clone 122, spanning all three TREs, cloned into pBLCAT3 (Bigler and Eisenman, 1994). pRSV-Pit-1 was a gift from H. H. Samuels (New York University Medical Center, New York, NY) and contains a cDNA clone of the rat Pit-1/GHF-1 gene linked to the RSV LTR (Fox et al., 1990). The RSh-TRβ construct contains the wild type human TRβ1 gene, and the RSh-TRβ C122>A expression plasmid contains an in vitro-generated mutant human TRβ1 gene (alanine instead of cysteine at position 122), both under control of the RSV LTR (Nelson et al., 1993). The RSh-
RXRα expression vector contains human retinoid X receptor α cDNA linked to the RSV LTR. ΔMTV-CAT contains a CAT reporter gene driven by the ΔMTV LTR promoter (Thompson and Evans, 1989). RSh-TRβ, RSh-TRβ C122>A, RSh-RXRα, and ΔMTV-CAT were gifts from P. J. Romaniuk. pRSh-TRα was obtained from M. Harkey (University of Washington, Seattle, WA) and contains the E. coli β-galactosidase gene under control of the RSV LTR (Gorman et al., 1983).

(2) Microinjections and ELISA

A lobe of ovary was surgically removed from an adult female Xenopus laevis and processed as described (Allison et al., 1991). Microinjections were performed according to published methods with modifications (Allison et al., 1991, 1993; Nagl et al., 1995). Stage V (Dumont, 1972) oocytes were microinjected with 20 nl plasmid DNA in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) into the nucleus by the 'blind' injection method with the needle inserted in the center of the animal pole (Gurdon and Melton, 1981; Colman, 1984). After microinjection, oocytes were incubated in O-R2 (Appendix 2; Allison et al., 1991) medium for 20-24 h at 18°C in the presence or absence of 100 nM T3 (3,5,3'-triiodo-L-thyronine, Sigma, Chemical Co., St Louis, MO). Healthy oocytes (uniform pigment, not mottled) were homogenized singly in 200 μl 0.25 M Tris buffer, pH 7.5, and centrifuged at 10,000 x g in a microfuge at 4°C for 10 min. The supernatant was then centrifuged as before. This extract was used in the determination of CAT and β-galactosidase protein expression levels by enzyme linked immunosorbant assay (ELISA) according to the manufacturer's specifications (5 Prime-3 Prime, Inc., Boulder, CO). The concentration of total protein in extracts from single oocytes was adjusted to a
concentration of 175 to 200 μg/ml by 1:1 dilution with Dilution Buffer. Microwells coated with rabbit polyclonal antibody specific to CAT protein or specific to the E. coli β-galactosidase protein (β-Gal) were incubated with extracts from microinjected oocytes, uninjected controls, and appropriate pure CAT or β-Gal protein standards. Biotinylated secondary antibody to CAT or β-Gal was then bound to the primary antibody-antigen complex. After washing, bound biotinylated antibody was quantitated colorimetrically by incubation with streptavidin-conjugated alkaline phosphatase and p-nitrophenyl phosphate as substrate. The wells were read at 405 nm against the reagent blank in a microtiter well reader (model EL 311, Bio-Tek Instruments, Winooski, VT). For each assay, a standard curve utilizing four pure protein standards was prepared, to ensure that CAT or β-Gal concentrations of sample extracts fell within the linear range of the assay. All experiments were repeated two to three times with different animals since there can be variability in transcriptional activity between different batches of oocytes. The data on reporter gene expression levels were analyzed by nested analysis of variance (ANOVA) controlling for variability between females and between independent ELISA assays.

(3) Immunoprecipitation of v-erbA protein

Oocytes were injected into the nucleus with 2.5 ng RS-v-erbA in 20 nl TE, pH 8.0. Oocytes were then incubated in a sterile microtitre plate (5 oocytes/well) in 30 μl of O-R2 with 1 mCi/ml L-[35S] methionine (1000 Ci/mmol, Amersham Pty Ltd., Auckland, New Zealand) at 18°C for 20-24 h. Twenty microliters of preswollen protein G-Sepharose beads (Gamma Bind Plus Sepharose, Pharmacia LKB Biotechnology, Auckland, New Zealand) in 0.45 ml NET-2 (Appendix 2; Allison et
al., 1991) was aliquoted per microfuge tube, followed by the addition of 30 μl of monoclonal antibody LA038 against v-erbA residues 58-75 (Quality Biotech, Camden, NJ). Antibodies were bound to protein G-Sepharose beads for 2 h at room temperature with end-over-end rotation. After incubation, the resin was pelleted for 5 sec in a microfuge and resuspended in 1 ml of NET-2, and the wash was repeated three times. Nuclear and cytoplasmic fractions of 20 v-erbA-injected oocytes per sample were prepared manually as described (Allison et al., 1991) after fixation in 1% ice-cold trichloroacetic acid for 5 min. 0.5 ml NET-2 with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) was added to both fractions. Twenty uninjected control oocytes were pooled per sample and homogenized in 0.5 ml NET-2 with 0.1 mM PMSF added. Cytoplasmic fractions and control homogenates were precleared of yolk and pigment by microfuging for 10 min at 10,000 x g at 4°C. The supernatants of control samples and cytoplasmic fractions, and the nuclear fraction samples, were added to each washed protein G-Sepharose-antibody pellet and incubated at 4°C for 1 h with end-over-end rotation. After incubation, the resin with bound antigen was pelleted, and washed four times with NET-2. Forty microliters of 2 X SDS sample buffer (4% SDS, 20% glycerol, 120 mM Tris, pH 6.8, 0.01% bromophenol blue) were added to each pellet, the samples were heat denatured to release the antigen and the resin was pelleted for 5 sec in a microfuge. The supernatant, with 1 mM dithiothreitol (DTT) added, was resolved by 12% SDS-polyacrylamide gel electrophoresis followed by fluorography.

(4) **ELISA for total T₃**

Ten oocytes per sample, either incubated in 100 nM T₃ for 20 h and subsequently
washed three times in O-R2, or cultured in O-R2 only, were homogenized in 100μl 0.25 M Tris buffer, pH 7.5, and centrifuged at 10,000 x g in a microfuge at 4°C for 10 min. The supernatant was then centrifuged as before. This extract was used in the quantitative determination of total T₃, both bound and free hormone, in oocytes by ELISA (Enzymun-Test T₃, Boehringer Mannheim, Auckland, New Zealand). Test tubes coated with streptavidin were incubated with oocyte extracts or T₃ standards, together with anti-T₃ antibody-horseradish peroxidase (POD) conjugate. T₃ standards were prepared in 0.25 M Tris buffer, pH 7.5, at 0, 0.1, 1, 10 and 100 nM. Binding of sample T₃ to anti-T₃ antibodies was then competed by addition of biotinylated T₃ polyhapten, which anchors previously unbound anti-T₃ antibodies to the streptavidin-coated test tube wall. After washing, bound anti-T₃ antibody-POD conjugate was quantitated colorimetrically by incubation with 2, 2'-azino-di-[3-ethyl-benzthiazoline sulfonate (6)] (ABTS)/H₂O₂. The samples were transferred to micro-cuvettes and read against buffer at 420 nm in a spectrophotometer. For each assay, a standard curve utilizing the five T₃ standards was prepared. Two independent determinations were carried out with oocytes from two different animals, and four replicates (10 oocytes/replicate) were assayed in each determination.

(5) Preparation of nuclear protein extracts from ovine anterior pituitary cells and cytoplasmic injection

Nuclear protein extracts were prepared essentially as described by Maxson et al. (1988). All procedures were carried out at 4°C. Ten milliliters of sheep anterior pituitary cells maintained in primary cell culture (4.8 x 10⁶ viable cells/ml) were concentrated by centrifugation, washed in phosphate-buffered saline, and then washed
once in 1.5 M glucose. The resultant partially lysed cells were homogenized gently in 10 volumes of low-salt buffer (4 mM magnesium acetate, 50 mM Tris, pH 7.4, 0.5% Nonidet P-40, 10 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]). Two to five strokes in a Dounce glass homogenizer were sufficient to complete lysis. The homogenate was overlaid (15 ml per tube) on step sucrose gradients consisting of 5 ml of 1.7 M sucrose on 5 ml of 2.2 M sucrose in 30-ml Nalgene tubes. The gradients were centrifuged at 5,000 x g for 15 min, and the gray nuclei were collected from the interface between the two sucrose solutions. The nuclei were diluted twofold with a buffer containing 75 mM NaCl, 25 mM EDTA, 5 mM Tris, pH 7.5, 20 mM 2-mercaptoethanol, and 0.1 mM PMSF. Partially lysed nuclei and chromatin were collected by centrifugation at 10,000 x g for 30 min. The chromatin-nuclei pellet was suspended in extraction buffer (0.45 M NaCl, 20 mM Tris, pH 7.0, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol [DTT], 0.1 mM PMSF), and vortexed for several minutes, and the insoluble material was collected by centrifugation at 10,000 x g for 30 min. The supernatant was set aside, and the extraction and centrifugation were repeated on the residual nuclear material with extraction buffer containing 1 M NaCl and then 2 M NaCl. The extracts of all three salt washes were pooled and concentrated by precipitation with ammonium sulfate (0.35 g/ml), dialyzed for 24 h against several changes of injection buffer (100 mM NaCl, 20 mM Tris, pH 7.0, 5 mM MgCl$_2$, 1 mM EDTA, 0.1 mM EGTA, 5% glycerol, 0.5 mM DTT, 0.1 mM PMSF), and concentrated severalfold by centrifugation in centricon 10 micro concentrators (Amicon, Beverly, MA). Extracts were stored frozen at -80°C. Five hours before microinjection of gene templates, oocytes were microinjected with 20 nl of extract into the cytoplasm.
3. Results

(1) Both constitutive and T₃-mediated gene activation functions of TRα and TRβ were induced in Xenopus oocytes and enhanced by RXR

I was interested to establish whether Xenopus oocytes can be used for the reconstitution of nuclear receptor networks in vivo. In this context, I wished to ascertain whether oocytes provide a cellular background for nuclear hormone receptor action similar to either yeast or mammalian cells, or whether they constitute a unique context. TR mRNA is present at all stages of oogenesis (Kawahara et al., 1991) and in fertilized eggs (Banker et al., 1991); however, at the onset of my study, TR protein had not been detected by immunoprecipitation or Western immunoblotting (Banker et al., 1991), suggesting that maternal transcripts are stored for use later in embryogenesis. Results obtained by enzyme linked immunosorbant assay (ELISA) showed that oocytes contain no detectable endogenous T₃ (< 0.1 nM). After incubation in 100 nM T₃, oocytes exhibited an intracellular hormone concentration of ~ 5 nM (Fig. 7), which falls within the physiological range for T₃ action. Thus, I presumed that Xenopus oocytes would be suitable for experiments investigating transcriptional regulation by exogenous TR and v-erbA protein at a range of diverse TREs, both with and without added T₃.

I sought to reflect the natural diversity of TREs by employing four positively regulated response elements possessing different half-site characteristics; i.e., the direct repeat TRE in the rat malic enzyme gene promoter (malTRE) (Petty et al., 1990), the inverted palindromic TRE in the promoter region of the chicken lysozyme gene (lysTRE) (Baniahmad et al., 1990), the tripartite TRE in the third intron of the
Figure 7. Determination of endogenous and exogenous T₃ in *Xenopus* oocytes by ELISA. A standard curve from ELISA of T₃ standards at the indicated concentrations was plotted against absorbance at 420 nm. The absorbance readings from ELISA of 10 pooled oocytes, cultured in O-R2 (endogenous) or incubated in 100 nM T₃ (exogenous) for 20 h were plotted on the standard curve to determine the T₃ content. The error bars indicate the SEMs of two independent determinations with oocytes from different females (4 replicates/treatment/determination).
rat growth hormone gene (rGH$_3$TRE) (Sap et al., 1990) and a synthetic palindromic TRE (TREp) (Damm et al., 1989); and one complex response element which is strongly induced by unliganded TR and repressed by T$_3$ in mammalian cells (clone 122 TREs) (Bigler and Eisenman, 1994). A rat TR$\alpha$ (rTR$\alpha$) expression plasmid and a CAT reporter gene under the control of one of five different TREs were microinjected into the oocyte nucleus. Unliganded rTR$\alpha$ induced transcription of all five TRE-regulated CAT reporter genes (Fig. 8). Constitutive reporter gene induction by rTR$\alpha$ was strongest when under the control of lysTRE, TREp, or the clone 122 TREs. CAT reporter gene transcription under control of three positively regulated TREs (malTRE, lysTRE, TREp) was further enhanced by the addition of T$_3$, but was unresponsive to hormone with rGH$_3$TRE (Fig. 8). In contrast to results obtained in CV1 (monkey kidney) cells (Bigler and Eisenman, 1994), addition of T$_3$ did not significantly repress TR-mediated transcription regulated by the clone 122 TREs (Fig. 8). Reporter gene induction by TR was shown to be dependent on the presence of a TRE in oocytes, as expression levels of the ΔMTV-CAT reporter gene lacking a TRE sequence were not induced by coinjection of rTR$\alpha$, in the presence or absence of T$_3$ (Fig. 8).

Unexpectedly, there was a weak T$_3$-dependent induction of the tk-TREp-CAT and TREp-ΔMTV-CAT reporter constructs in the absence of exogenous TR, while the ΔMTV promoter alone was not responsive to T$_3$ (Fig. 8). Significant induction from TREp in response to T$_3$ was observed in samples analyzed 4 h after microinjection of the reporter gene into the nucleus and persisted over a 36 h period of hormone incubation (Fig. 9). Contrary to my original assumptions, these results suggested the presence of low levels of an endogenous TR-like activity in oocytes. A recent study
Figure 8. TR-mediated TRE-CAT reporter gene transcription in *Xenopus* oocytes. Two ng ΔMTV-CAT or *tk*-CAT reporter gene construct linked to one of five different TREs as indicated, or 2 ng ΔMTV-CAT lacking a TRE, was microinjected into the oocyte nucleus together with 0.5 ng of the rTRα expression vector. Oocytes were cultured in the presence or absence of 100 nM T₃ (TR/+T3 and TR/-T3) for 24 h. Alternatively, oocytes were microinjected with reporter constructs alone, and incubated with and without hormone (+T3/-T3). ΔMTV-TREp-CAT was tested with and without T₃ only. Activation of the reporter gene was measured in terms of the levels of CAT protein present in oocytes, analyzed individually by ELISA. Basal transcription levels of the reporter constructs in the absence of exogenous TR and T₃ were arbitrarily assigned the value 1. For each CAT reporter construct 2 or 3 independent experiments (5 oocytes per treatment) were performed and the mean fold induction over basal transcription was calculated. The error bars indicate the SEMs. Differences in levels of reporter gene transcription were significant at p < 0.0001.
Figure 9. Time course of transcriptional activation of the tk-TREp-CAT reporter construct by endogenous TR in the presence of T₃. Two ng tk-TREp-CAT reporter construct was microinjected into the oocyte nucleus. Oocytes were then cultured with and without 100 nM T₃. Activation of the reporter gene was measured in terms of the levels of CAT protein present in single oocytes at the indicated times after microinjection, and expressed in arbitrary units based on the measurement of CAT levels by ELISA. Five oocytes per time point were assayed and the time course was repeated 4 times. The error bars indicate the SEMs.
has now reported that *Xenopus* oocytes contain both TRα and TRβ protein (Eliceiri and Brown, 1994). The observed T₃-mediated induction of a CAT reporter gene under control of TREp in the absence of exogenous TR indicates that these endogenous TRs can function as T₃-regulated activators.

The β isoform of the receptor, human TRβ (hTRβ), also constitutively induced transcription at TREp, albeit to a lesser degree than rTRα (Fig. 10). Gene activation by hTRβ was not significantly enhanced in the presence of hormone at TREp. Moreover, to achieve detectable levels of reporter gene induction by hTRβ, a five times greater amount of the expression vector (RSh-TRβ) had to be microinjected compared to RS-rTRα. This increased requirement might have been due to differences in the expression of the two constructs in oocytes, or to the decreased binding affinity of TRβ for palindromic TREs (Privalsky *et al.*, 1990; Holloway *et al.*, 1990).

RXRs have recently been shown to heterodimerize with TRs, enhancing TR binding to TRE elements, and augmenting T₃-mediated transcriptional activation (reviewed in Mangelsdorf and Evans, 1992). Therefore, it was of interest to test how heterodimerization with RXR affects the action of TR in oocytes. In this series of experiments, equimolar amounts of expression plasmids for human RXRα (hRXRα) and rTRα, or hTRβ, were microinjected into the oocyte nucleus, together with the tk-TREp-CAT reporter construct. Employing TREp in this experiment allowed a direct comparison between activation by both TR and RXR with induction by TR alone, because TREp binds both TR monomers and homodimers, as well as RXR/TR heterodimers (Forman *et al.*, 1992, 1995b). As shown in Fig. 10, hRXRα enhanced constitutive reporter gene activation by both rTRα and hTRβ.
Figure 10. tk-TREp-CAT reporter gene transcription in the presence of hRXRα and rTRα or hTRβ. Oocytes were microinjected with 2 ng tk-TREp-CAT only (reporter only), or together with varying amounts of hRXRα (RXR), rTRα (TRalpha) and hTRβ (TRbeta) expression plasmids as indicated. Oocytes were incubated with and without hormone (+T3, -T3) for 24 h, and then analyzed individually by ELISA as described in Figure 8.
reporter only
2.5ng RXR
0.5ng TRalpha
0.5ng TRalpha/0.5ng RXR
0.5ng TRbeta
0.5ng TRbeta/0.5ng RXR
2.5ng TRbeta
2.5ng TRbeta/2.5ng RXR

fold induction

0 1 2 3 4 5 6 7 8 9

reporter only
2.5ng RXR
0.5ng TRalpha
0.5ng TRalpha/0.5ng RXR
0.5ng TRbeta
0.5ng TRbeta/0.5ng RXR
2.5ng TRbeta
2.5ng TRbeta/2.5ng RXR

+ - 1 - 1

-13 -13
Coexpression of hRXRα increased T₃-independent and T₃-mediated reporter gene activation by rTRα to the same level. The presence of hRXRα enhanced the constitutive activator function of hTRβ (2.5 ng expression vector microinjected) to levels seen with unliganded rTRα alone (0.5 ng expression vector). In addition, with hRXRα present, hTRβ activated the reporter gene in a T₃-responsive manner to the same level as rTRα.

To summarize, both rTRα and hTRβ constitutively activated reporter gene transcription at five different TREs, while activation was further inducible by T₃ at a subset of TREs. Furthermore, coexpression of hRXRα enhanced both ligand-independent and ligand-dependent activation by rTRα and hTRβ at TREp. An endogenous TR activity mediated weak T₃-induction of TREp.

(2) **Only certain naturally occurring TREs acted as control elements for repression by v-ErbA in the presence or absence of T₃**

In mammalian and avian cells, TR activities are blocked by v-ErbA acting as a dominant repressor at the level of the TRE. However, this function of v-ErbA is not universal, but depends on cellular context (Privalsky et al., 1990; Smit-McBride and Privalsky, 1993). Potent suppression of basal transcription of T₃-regulated genes in the absence of TR and T₃ has also been shown in mammalian cells (Damm et al., 1989; Damm and Evans, 1993), and has been implicated in v-erbA oncogenesis (Fuerstenberg et al., 1992; Privalsky, 1992; Sharif and Privalsky, 1991; Zenke et al., 1990). I sought to establish the action of v-ErbA in *Xenopus* oocytes in two ways. The first series of experiments tested the competitive interaction between v-ErbA and rTRα at naturally occurring TREs, by determining the extent to which v-ErbA was
able to dominantly repress TR-induced activation of the reporter gene constructs.

The second set of experiments tested the ability of v-ErbA to suppress basal promoter activity of the reporter genes in the absence of TR and T₃.

Prior to experiments, I determined whether the v-erbA product is transcribed and translated in Xenopus oocytes, and whether it is targeted to the nucleus. Nuclear and cytoplasmic fractions were prepared manually for immunoprecipitation assays. The v-erbA expression plasmid was shown to direct the synthesis of a 75 kDa polypeptide which was predominantly recovered from the nuclear fraction using a monoclonal anti-v-erbA antibody (Fig. 11). This polypeptide is identical in size to that expected for the gag-v-erbA protein (Privalsky, 1992). This indicates that a full-length v-erbA product is synthesized in oocytes. Furthermore, the immunoprecipitation assays showed that the v-erbA product is correctly targeted to the nucleus in oocytes.

Having determined that v-erbA is expressed in Xenopus oocytes, I then carried out a series of coinjection experiments to test the dominant repressor function of v-ErbA. In both the presence and absence of T₃, v-ErbA acted as a dominant repressor of rTRα-mediated CAT reporter gene transcription from lysTRE, malTRE, and TREp (Fig. 12A). Repression was most complete at TREp. The dominant action of v-ErbA was dose-dependent, as a 5-fold excess of the v-erbA expression plasmid produced an even greater repression of TR-mediated induction of the reporter genes than an equal ratio of v-erbA and TR templates (data not shown). In contrast, v-ErbA did not repress rTRα-mediated transcription under the control of rGH₃TRE (Fig. 12A). However, coexpression of the cofactor hRXRα reduced transcription in the presence of rTRα and v-ErbA at this TRE (Fig. 12A).

In mammalian cells, v-erbA represses basal transcription of T₃-responsive genes in
Figure 11. Immunoprecipitation of v-erbA protein from cytoplasmic and nuclear fractions. Oocytes microinjected with 2.5 ng v-erbA expression vector, and un.injected control oocytes, were cultured in O-R2 medium with 1 mCi/ml L-[35S]methionine for 20 to 24 h. Nuclei of v-erbA-injected oocytes were then manually isolated. Cytoplasmic (Cy) and nuclear (N) fractions of 20 v-erbA-injected oocytes, and homogenates of 20 un.injected whole oocytes (C), were incubated with protein G-Sepharose-antibody complexes in an immunoprecipitation assay. Labelled polypeptides were recovered and separated electrophoretically on 12% SDS-polyacrylamide gels followed by fluorography. The 75 kDa gag-v-erbA protein (Privalsky, 1992) was predominantly immunoprecipitated from the nuclear fraction.
the absence of T₃ (Damm et al., 1989; Damm and Evans, 1993). In the present study, v-ErbA did not act as a repressor of basal gene transcription under control of four different positively regulated TREs in oocytes (Fig. 12A). Repression of basal transcription by v-ErbA would have been detectable by the CAT ELISA employed, since the linear range of the assay (tested down to A₄₀₅ ~0.15) extended below the CAT protein levels observed due to basal promoter activity (A₄₀₅ ~0.23-0.3) (data not shown). The loss of this function by v-ErbA parallels the lack of repression of basal transcription by unliganded exogenous TR in oocytes. Importantly, expression of v-erbA in oocytes abolishes its function as a repressor of basal promoter activity, but does not interfere with its dominant repressor potential.

I showed that dominant repression by v-ErbA in the oocyte is a specific effect in the following ways. First, transcription levels of the ΔMTV-CAT reporter plasmid were unaffected by the presence of rTRα and/or v-ErbA with and without T₃, showing that gene regulation by rTRα and v-ErbA is dependent on the presence of a TRE (Fig. 12A). Second, T₃-dependent induction by rTRα of the ΔMTV-lysTRE-CAT reporter gene was unaffected by the coinjection of the lacZ expression vector in place of the v-erbA construct (Fig. 12B). Both in the presence and absence of the lacZ vector, T₃ enhanced reporter gene expression 1.5 fold over levels induced by unliganded rTRα. This control was performed at a 2-fold greater concentration of the lacZ vector (5 ng) compared with experiments using v-erbA construct (2.5 ng). Dominant repression by v-ErbA was thereby shown not to be caused by 'non-specific squelching' (Ptashne, 1988); i.e., essential transcription factors are not simply being titrated by the presence of an excess of v-erbA expression template.
**Figure 12.** Effects of v-ErbA on TRE-CAT reporter gene transcription. (A) Dominant repression of TR-mediated TRE-CAT reporter gene transcription by v-ErbA in *Xenopus* oocytes. Oocytes were cultured in the presence or absence of 100 nM T₃ after microinjection with 2 ng ΔMTV-CAT or tk-CAT reporter gene construct linked to four different TREs as indicated, or 2 ng ΔMTV-CAT without a TRE, and 0.5 ng rTRα expression vector alone (TR/+T₃, TR/-T₃), or together with a 5-fold excess of v-erbA (2.5 ng) (v-erbA/TR/+T₃, v-erbA/TR/-T₃). Two ng rGH₃TRE reporter construct plus 0.5 ng rTRα, 2.5 ng v-erbA, and 2.5 ng hRXRα expression plasmids were also tested (v-erbA/TR/RXR/-T₃, v-erbA/TR/RXR/+T₃). In addition, 2 ng TRE-CAT reporter gene construct was microinjected into the oocyte nucleus with 2.5 ng v-erbA expression plasmid, and oocytes were cultured without T₃ (v-erbA/-T₃). Alternatively, oocytes were microinjected with reporter constructs alone, and incubated in the absence of T₃ (-T₃). After 24 h, oocytes were assayed individually for CAT protein by ELISA. Basal transcription rates of the CAT reporter constructs in the absence of exogenous TR, v-ErbA and T₃ (-T₃) were arbitrarily assigned the value 1. Two or 3 independent experiments (5 oocytes per treatment) were performed for each TRE-CAT reporter gene construct and the mean fold induction over basal transcription was calculated. The error bars indicate the SEMs. Differences in levels of reporter gene transcription were significant at p < 0.0001. (B) T₃-induction of lysTRE-CAT reporter gene transcription in the presence of rTRα and lacZ expression plasmids. Oocytes were microinjected with 2 ng ΔMTV-lysTRE-CAT reporter gene construct plus 0.5 ng rTRα with or without 5 ng lacZ expression vector, as indicated, and incubated in the presence or absence of 100 nM T₃ (-T₃/+T₃). A single experiment with 5 oocytes per treatment was performed and analyzed as described for (A). (C) Constitutive trans-activation by v-ErbA at the clone 122 TREs. Oocytes were cultured with and without T₃ after microinjection of 2 ng pTPT-CAT plasmid, containing the clone 122 TREs linked to a tk-CAT reporter construct (tk-clone 122), or 2 ng ΔMTV-lysTRE-CAT plasmid, and 0.5 ng v-erbA expression vector (v-erbA/+T₃, v-erbA/-T₃). Alternatively, oocytes were microinjected with reporter constructs alone, and incubated in the absence of T₃ (-T₃). Three independent experiments with 5 oocytes per treatment were performed and analyzed as described for (A).
The v-erbA protein binds T₃ only at very low affinity, due to several mutations in the C-terminal domain (Damm et al., 1989; Forrest et al., 1990; Privalsky et al., 1988; Sap et al., 1989). Presumably, this lack of hormone binding would render v-ErbA highly resistant to undergoing a ligand-induced conformational change. For this reason, I was interested to ascertain how v-ErbA acts at the clone 122 TREs in oocytes. In mammalian cells, T₃ inhibits the activator function and induces the repressor function of TR at this TRE (Bigler and Eisenman, 1994). I showed in the previous section that, in oocytes, TR constitutively activated transcription at the clone 122 TREs. I also observed constitutive trans-activation of the reporter gene construct by v-ErbA (Fig. 12C). This induction by v-ErbA was not significantly affected by T₃. The v-erbA protein was also unresponsive to T₃ at lysTRE in oocytes (Fig. 12C).

In the previous section I reported that there was a weak T₃-dependent induction of the TREp-CAT reporter gene in the absence of exogenous TR, suggesting the presence of low levels of T₃-regulated endogenous TR in oocytes. The T₃-dependent reporter gene induction by endogenous TR was dominantly repressed to the same level by v-ErbA and a dominant negative in vitro-generated mutant of human TRB (TR C122>A) (Fig. 13). This DNA-binding deficient mutant is thought to repress TR-mediated gene transcription by forming inactive dimers with TR (Nelson et al., 1993). Therefore, dominant repression by TR C122>A suggests that endogenous TR was able to dimerize with the exogenous mutant TRB. The slight T₃-dependence of transcription in the presence of TR C122>A was interpreted as not biologically significant, because of high sample variability. Repression of the reporter gene by v-ErbA or TR C122>A was dependent on the presence of a TRE. The lacZ reporter gene, which lacks a TRE, was expressed at levels similar to those of the CAT
Figure 13. Dominant repression of an endogenous TR-like activity by v-ErbA or an in vitro-generated mutant of TR. Oocytes were microinjected with 2 ng of tk-TREp-CAT reporter plasmid alone (none), or together with v-erbA or TR C122>A expression plasmids in the presence or absence of 100 nM T3 (+T3, -T3) in the combinations indicated. As a control, 2 ng RSV-lacZ reporter gene instead of tk-TREp-CAT construct was coinjected with TR C122>A or injected alone (none). Relative reporter gene transcription was expressed in arbitrary units based on measurements of CAT or β-galactosidase protein levels by ELISA. Two independent experiments with 5 oocytes per treatment were performed. The error bars indicate the SEMs. Transcription levels of the RSV-lacZ construct did not differ significantly between treatments (p < 0.39). Transcription levels of the tk-TREp-CAT construct in the presence or absence of T3 were significantly different at p < 0.0001.
RSV-lacZ

- +3ng TR C122>A
- none

- +3ng TR C122>A
- +3ng v-erbA
- none

tk-TREp-CAT

Relative reporter gene transcription
reporter gene, and was not repressed by either v-ErbA (data not shown) or TR C122>A (Fig. 13).

In summary, my results show that v-ErbA displays an altered pattern of activity in oocytes, compared to both mammalian cells and the yeast, S. cerevisiae. v-ErbA did not repress basal reporter gene transcription, yet acted as a dominant repressor of TR action at the positively regulated TREs tested, with the exception of rGH3TRE. At the clone 122 TREs, v-ErbA acted as a constitutive activator.

(3) Pituitary-specific transcriptional regulation of rGH3TRE by rTRα and v-ErbA

In a previous section, I showed that the T3-dependent activator function of rTRα was not induced at rGH3TRE in the presence of hormone (Fig. 8). Previous studies have shown that optimal transcription of the rat growth hormone gene (rGH) under control of TRE sequences in the promoter region is dependent on a cell-specific factor, Pit-1/GHF-1, binding at upstream elements (Lefevre et al., 1987). Since the rGH gene is expressed only in somatotrophic cells of the anterior pituitary gland, gene regulation at rGH3TRE, the TRE from the third intron, presumably also requires cell-specific auxiliary factors, of known or unknown origin. For this reason, I sought to reconstitute the nuclear environment of anterior pituitary cells in oocytes. Xenopus oocytes possess the experimentally attractive feature of correctly targeting nuclear proteins, injected into the oocyte cytoplasm, to the nucleus (see, for example, Maxson et al., 1988). In this experiment, nuclear protein extract from ovine anterior pituitary cells was injected into the oocyte cytoplasm five hours before nuclear microinjection of gene templates.
When oocytes were microinjected with nuclear protein extract, both the hormone-independent and hormone-dependent activator functions of rTRα were induced at rGH3TRE (Fig. 14A). Surprisingly, under the same conditions, v-ErbA acted as a constitutive activator at rGH3TRE, whose strength of activation exceeded that of rTRα (Fig. 14A). Increasing the amount of v-erbA expression template injected did not enhance this response further (data not shown). In contrast, v-ErbA did not affect basal promoter activity of this TRE-reporter construct in the absence of nuclear protein extract (Fig. 14A).

These findings suggested that auxiliary factor(s), present in the injected nuclear protein extract from anterior pituitary cells, significantly altered the effect of both rTRα and v-ErbA on reporter gene regulation at rGH3TRE in oocytes. Two plausible candidates for this auxiliary role are the pituitary-specific transcription factor Pit1/GHF-1 and RXR. To test this possibility, expression vectors for either Pit-1/GHF-1 or hRXRα were coinjected with rTRα or v-erbA constructs, and reporter gene transcription regulated by rGH3TRE was assayed. Transcription was weakly induced by hRXRα alone, while Pit-1/GHF-1 had no significant effect on basal promoter activity (Fig. 14B). The activity patterns of rTRα and v-ErbA observed in the presence of nuclear protein extract of anterior pituitary cells were not replicated in the presence of hRXRα or Pit-1/GHF-1 (Fig. 14B). Instead, coexpression of hRXRα or Pit-1, together with rTRα, reduced trans-activation of rGH3TRE (Fig. 14B) compared to levels of trans-activation by rTRα alone (cf. Figs. 12A and 14B). Pit-1 repressed induction of rGH3TRE by liganded rTRα below basal transcription (Fig. 14B). Compared to levels in the presence of v-ErbA only (Fig. 14A), coexpression
Figure 14. Pituitary-specific transcriptional regulation of rGH3TRE by rTRα and v-ErbA. (A) Action of rTRα and v-ErbA at rGH3TRE in the presence of pituitary nuclear extract from ovine anterior pituitary cells. Oocytes were either microinjected with 20 nl of nuclear protein extract into the cytoplasm five hours before nuclear injection of gene templates (+PNE) or microinjected with the gene templates in the absence of nuclear protein extract (-PNE). Oocytes were then cultured in the absence or presence of 100 nM T3 (-T3, +T3) after microinjection with 2 ng ΔMTV-rGH3TRE-CAT reporter gene construct alone (reporter only), or together with 0.5 ng rTRα (plus TR) or v-erbA expression vector (plus v-erbA). After 24 h, oocytes were assayed individually for CAT protein by ELISA. Basal transcription rates of the CAT reporter construct in the absence of nuclear protein extract, exogenous TR, v-ErbA and T3 (reporter only, -PNE, -T3) were arbitrarily assigned the value 1. (B) Action of rTRα and v-ErbA at rGH3TRE in the presence of hRXRα or Pit-1/GHF-1. Oocytes were microinjected with 2 ng ΔMTV-rGH3TRE-CAT reporter gene construct only (reporter only, no co-factor), or together with 2.5 ng expression vector for either hRXRα (+RXR) or Pit-1/GHF-1 (+Pit-1). Alternatively, 0.5 ng expression plasmid for rTRα (plus TR) or v-erbA (plus v-erbA) were microinjected with ΔMTV-rGH3TRE-CAT, and 2.5 ng expression vector for hRXRα (+RXR) or Pit-1/GHF-1 (+Pit-1) was added. Oocytes were cultured with and without T3 (+T3, -T3). After 24 h, oocytes were assayed individually for CAT protein by ELISA. Basal transcription rates of the CAT reporter construct in the absence of co-factors and T3 (reporter only, no co-factor, -T3) were arbitrarily assigned the value 1. (A) and (B) Three independent experiments (5 oocytes per treatment) were performed and the mean fold induction over basal transcription was calculated. The error bars indicate the SEMs.
of hRXRα with v-ErbA induced transcription slightly, while Pit-1 had no effect (Fig. 14B). With hRXRα present, transcription was induced equally by rTRα or v-ErbA. These effects were not further investigated, since this series of experiments was carried out to test hRXRα and Pit-1 as potential candidates for conferring positive T₃-responsiveness on rTRα, and strong constitutive activator function on v-ErbA, at rGH₃TRE. Taken together, these results suggest that auxiliary factor(s), other than RXR and Pit-1/GHF-1, present in nuclear protein extracts of anterior pituitary cells, are necessary for T₃-induction of rGH₃TRE by rTRα and conversion of v-ErbA into a constitutive activator at this TRE.

4. Discussion

(1) Both α and β isoforms of TR act as constitutive activators in oocytes

In Xenopus oocytes, rTRα acted as a ligand-independent transcriptional activator at four positively regulated TREs (lysTRE, malTRE, TREp and rGH₃TRE). With the exception of rGH₃TRE, addition of T₃ led to a further increase in CAT reporter gene expression by rTRα. The 3.4 to 7-fold T₃-dependent reporter gene induction observed in oocytes was comparable to activation in mammalian cells (Banaihamad et al., 1992; Damm et al., 1989; Hermann et al., 1993). hTRβ also acted as a weak hormone-independent activator at TREp, but was not further inducible by hormone. The observed differences in ligand-independent activation function between rTRα and hTRβ are most likely due to structural divergence in the N-terminal domain (Glass and Rosenfeld, 1991).
Both ligand-independent and ligand-induced activation functions (Barettino et al., 1994; Bigler and Eisenman, 1994; Forman et al., 1988; Forman and Samuels, 1990; Saatcioglu et al., 1993a) exhibit distinct cell type and promoter context specificity in TR and other receptors belonging to the steroid/thyroid hormone receptor gene superfamily (Privalsky et al., 1990; reviewed in Gronemeyer, 1992). Constitutive trans-activation by TR has also been shown in the yeast, S. cerevisiae (Privalsky et al., 1990; Smit-McBride and Privalsky, 1993), and in in vitro transcription assays using extracts from NT2 embryonal carcinoma cells or Namalwa B cells (Lee et al., 1994a). Taken together with these results, the ligand-independent activation function of mammalian TRs in oocytes points to a possible requirement of inhibitory auxiliary factor(s) for transcriptional repression. The finding that gene activation by unliganded TR at the clone 122 TREs was not reversed by T, in oocytes, while being suppressed by ligand in CV1 cells (Bigler and Eisenman, 1994), also points to a requirement for an inhibitory auxiliary factor for the suppression of trans-activation by TR.

I have shown that hRXRα enhances both ligand-independent and ligand-induced activation functions of rTRα and hTRβ at TREp, in analogy to the enhancement of both activation functions of hTRβ by hRXRα seen in the yeast (Lee et al., 1994b). Thus, RXRα is not the inhibitory factor in question. My results are in close agreement with recent evidence which suggests that an inhibitory factor suppresses trans-activation by unliganded TR in mammalian cells (Banaihmad et al., 1995; Casanova et al., 1994), and which also showed that RXR was not the major inhibitory factor in two cell lines tested (Casanova et al., 1994). More recently, two mammalian corepressors of TR and v-ErbA, N-CoR and SMRT, were identified by
two-hybrid screening in yeast (Chen and Evans, 1995; Hörlein et al., 1995; Kurokawa et al., 1995). N-CoR and SMRT interact with the hinge domain of unliganded TR and v-ErbA to mediate transcriptional silencing, and are released from TR upon ligand binding. However, corepressor release by ligand occurred only at positive TREs, and it is presently unknown whether N-CoR and SMRT are involved in transcriptional repression by liganded TR at negative TREs. Thus, either N-CoR, SMRT, or a related corepressor, most likely constitutes the component missing in oocytes that is required for transcriptional repression by unliganded rTRα, hTRβ and v-ErbA at positive TREs, and possibly also for repression by liganded rTRα at the clone 122 TREs.

The present study demonstrated that mammalian rTRα/hRXRα and hTRβ/hRXRα heterodimers constitutively activate transcription from a palindromic TRE linked to the viral tk promoter in Xenopus oocytes. Interestingly, heterodimers of Xenopus specific TRs (xTRs) and RXRs (xRXRs), expressed from microinjected mRNAs in Xenopus oocytes, strongly repress the T₃-inducible promoter of the Xenopus TRβA (xTRβA) gene in the absence of T₃, and efficiently induce transcription in response to T₃ (Wong and Shi, 1995; Wong et al., 1995). The 1.6 kb xTRβA promoter sequence contains a DR4 TRE which is located within the transcribed region, several other TREs with diverse half-site characteristics in the 5' region, and extensive flanking regions (Machuca et al., 1995; Ranjan et al., 1994; Wong et al., 1995). These elements might act in concert to mediate repression and activation by xTR/xRXR. The differential mode of action of mammalian and Xenopus TR/RXR heterodimers, at the TREp-tk and xTRβA promoters respectively, serves to highlight the importance of the chromatin environment around TRE binding sites for transcriptional regulation.
by TR (Almouzni and Wolffe, 1993a; Wong and Shi, 1995; Wong et al., 1995). In addition, and presumably unlike their mammalian counterparts, unliganded xTR/αRXR heterodimers might be able to directly inhibit the oocyte transcription machinery without requiring a corepressor(s).

(2) The v-erbA protein displays a novel pattern of activity in oocytes

Conversion of TR and v-ErbA into ligand-independent transcriptional activators has been shown to be mediated either by cell-specific protein-protein interactions (Forman et al., 1988; Privalsky et al., 1990), or by direct DNA binding (Saatcioglu et al., 1993a; Bigler and Eisenman, 1994). A constitutive activation function has also been identified in the N-terminal region of v-ErbA (Saatcioglu et al., 1993a). Under conditions that induce the constitutive activation function of TR, v-ErbA also acts as a constitutive activator in yeast (Privalsky et al., 1990), and in mammalian cells (Saatcioglu et al., 1993a). In contrast, I show here that the oocyte-specific cellular background induces the ligand-independent activation function of rTRα, but not of v-ErbA, at four positive TREs, suggesting that induction of this function can be differentially controlled in TRα and v-ErbA within the same cellular context. On the other hand, both rTRα and v-ErbA constitutively activated reporter gene transcription at the complex clone 122 TREs (Fig. 15). This TRE belongs to the same class of response elements as the TRE found in the Rous sarcoma virus-long terminal repeat, which mediates strong T3-independent activation by TR and v-ErbA in HeLa cells (Saatcioglu et al., 1993a). Binding to this type of TRE induces a conformational change and renders the constitutive N-terminal activation domain accessible to the transcription machinery (Saatcioglu et al., 1993a). In summary, expression of TR
Figure 15. TRE-modulated action of v-ErbA in oocytes. The identity of the nucleotide residue at the fourth position of the two primary half-sites in rGH$_3$TRE is indicated (boxed italicized letter) (Chen et al., 1993). Intervening nucleotides between half-sites are indicated by numbers. Arrows indicate orientation of half-sites. Differential levels of dominant repression are expressed in relative terms; (+++) indicates complete repression, (++) indicates moderate repression, and (+) indicates weak repression by v-ErbA.
TRE-modulated action of v-ErbA in oocytes:

1. Dominant repression

<table>
<thead>
<tr>
<th>TRE</th>
<th>AGGTCA</th>
<th>TGACCT</th>
<th>Strength of repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>tre1</td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>ma/tre</td>
<td>GGGTTA</td>
<td>AGGACA</td>
<td>++</td>
</tr>
<tr>
<td>ly/tre</td>
<td>TGACCC</td>
<td>AGGTCA</td>
<td>+</td>
</tr>
</tbody>
</table>

2. No repression of TR function by v-ErbA alone

<table>
<thead>
<tr>
<th>trans TRE</th>
<th>AGGTAA</th>
<th>AGTCCC</th>
<th>AGGTCA</th>
<th>(++; RXR corequirement)</th>
</tr>
</thead>
</table>

3. Activation

<table>
<thead>
<tr>
<th>clone 122 TREs</th>
<th>TAAGCCCGCGCGCGAGAAGGACGCCCCAAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREC1</td>
<td>ATACCTTATACCTCATGTAATAAG</td>
</tr>
<tr>
<td>TREC2</td>
<td>TCCGAGTGGACTCAGGCTGTCATGGGTG</td>
</tr>
</tbody>
</table>
and v-ErbA in oocytes induced the constitutive activation function only in TR at positive TREs, whereas this function was activated in both TR and v-ErbA at the clone 122 TREs in the same cellular context. It follows that the responses of the ligand-independent activation domains of TR and v-ErbA to cell-specific and TRE-mediated induction are not equivalent.

In oocytes, v-ErbA did not repress basal promoter activity under control of all four positive TREs tested (TREp, lysTRE, malTRE, and rGH3TRE). Like TR, the v-erbA protein possesses a repressor function located in the hinge domain (Damm and Evans, 1993). Transcriptional repression by TR is observed on minimal promoters containing a TATA box and TRE sequence (Baniahmad et al., 1990, 1992), and cell-free transcription assays have shown that unliganded TR functions as an active repressor of T3-responsive genes by inhibiting the formation of a functional preinitiation complex (Fondell et al., 1993). Presumably, repression by TR and v-ErbA occurs by a similar mechanism, namely, by interference with general transcription factors. In oocytes, protein-protein interactions between the repressor domain of v-ErbA and the general transcription machinery might be prevented by a lack of auxiliary inhibitory factor(s) or cell-specific characteristics of the general transcription complex.

(3) Dominant negative repression by v-ErbA depends on the nature of the TRE

The action of v-ErbA at rGH3TRE has not been tested in mammalian cells to my knowledge. In oocytes, v-ErbA failed to repress TR-mediated induction at rGH3TRE. At the same time, v-ErbA dominantly repressed trans-activation by TR at three other positive TREs with diverse half-site characteristics (malTRE, lysTRE and TREp).
The TRE-modulated action of v-ErbA in oocytes is summarized in Fig. 15. Although both primary half-sites of rGH3TRE possess a favorable thymidine nucleotide at the fourth position (Chen et al., 1993; Sap et al., 1990), and the sequence of the major binding site is identical to the AGGTC/GA consensus motif (Fig. 15), it appears that v-ErbA does not successfully compete with rTRα for binding due to the structure of rGH3TRE. As shown by other workers, heterodimerization with RXR-related factors can be essential for the dominant negative action of v-ErbA (Chen and Privalsky, 1993; Hermann et al., 1993). In the present study, a reduced level of trans-activation of rGH3TRE by rTRα and v-ErbA was observed in the presence of hRXRa. However, the significance of this finding is presently unclear, since hRXRa also reduced trans-activation by rTRα alone at this TRE.

4. An activity in nuclear protein extracts from anterior pituitary cells conferred T3-responsiveness to rTRα and converted v-ErbA into a constitutive activator at rGH3TRE

My findings suggest that T3-induction of rGH3TRE is regulated in a cell-specific manner. In the presence of nuclear protein extract from anterior pituitary cells, the T3-dependent activation function of rTRα was induced at rGH3TRE in addition to T3-independent trans-activation by rTRα without extract. I showed that T3-responsiveness of rTRα was not mediated by hRXRa or the pituitary-specific transcription factor Pit-1/GHF-1 which is required for efficient T3-induction of the growth hormone gene in somatotrophs (Lefevre et al., 1987). General mammalian-type transcription factors might have been involved in conferring T3-responsiveness to rTRα, since rGH3TRE is also T3-inducible in CV1 cells (Sap et al., 1990). The
tripartite rGH₃TRE, although located in the third intron of the rat growth hormone
gene, exhibits a markedly higher affinity for TR, as well as greater gene induction
ability, than the TRE located in the promoter region of the gene, indicating an
important in vivo role for this TRE sequence (Sap et al., 1990). Further indications
of the importance of regulatory regions in introns are the presence of a
glucocorticoid-responsive element in the first intron of the human growth hormone
gene (Slater et al., 1985), and the requirement for the presence of introns in the rat
growth hormone gene for efficient expression in transgenic animals (Brinster et al.,
1988). Interestingly, v-ErbA acted as an efficient constitutive activator at rGH₃TRE
in the presence of nuclear protein extract from anterior pituitary cells, whereas it had
no effect on basal promoter activity under the control of rGH₃TRE without extract.
Coexpression of Pit-1/GHF-1 or hRXRα did not replicate the mode of action of the
v-erbA protein as a constitutive activator seen with the nuclear protein extract. This
finding suggests that indirect or direct interactions with an as yet unidentified nuclear
factor present in pituitary cells result in a conformational change in v-ErbA at
rGH₃TRE that induces its constitutive activation function. The conversion of v-ErbA
into a constitutive activator by nuclear protein extract from anterior pituitary cells at
rGH₃TRE suggests that the dominant negative phenotype of the v-erbA oncogene can
be abolished by either direct or indirect interactions with other nuclear factors. My
experimental approach illustrates how studies of exogenous receptors in oocytes, in
conjunction with microinjected auxiliary proteins, may contribute to the
characterization of cell-specific and TRE-mediated mechanisms that modulate the
action of receptor domains.
III.

The v-erbA Oncogene Initiates Early and Intermediate Events of Meiotic Maturation in Xenopus Oocytes

1. Introduction

This chapter presents evidence to suggest that a direct link may exist between partial growth factor autonomy conferred by v-ErbA and induction of gene(s) normally controlled by mitogenic signalling networks. I show that v-ErbA can initiate the release from G₂ cell cycle arrest in stage VI (Dumont, 1972) Xenopus oocytes by activating gene expression. Ultrastructural analysis of v-erbA-expressing oocytes revealed transformations of the nuclear membrane indicative of early and intermediate stages of meiosis, while chromosome condensation and spindle formation did not occur. A portion of v-ErbA was localized to the cytoplasmic fibrils of the nuclear pore complexes, which raises the possibility that v-ErbA might affect the nucleocytoplasmic transport of a cell cycle regulator.

At the beginning of the introduction, the present model of growth-promotion by v-ErbA is reviewed. The following sections describe the regulation of meiotic pathways by progesterone or by introduced oncogenic proteins, and the

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Abbreviations used in this chapter: AL, annulate lamellae; AP-1, activator protein-1; GVM, germinal vesicle migration; GVBD, germinal vesicle breakdown; IBMX, isobutylmethylxanthine; MPF, maturation-promoting factor; MAPK, mitogen-activated protein kinase; NPC, nuclear pore complex; NLS, nuclear localization signal; RAR, retinoic acid receptor; TR, thyroid hormone receptor.
accompanying structural changes in the nucleus and the nuclear membrane. In
addition, the structure of nuclear pore complexes and their function in
nucleocytoplasmic transport is illustrated.

(1) **Deregulation of proliferative controls by v-ErbA**

Neoplastic transformation is mediated by the disruption of the signalling networks
which regulate cellular proliferation and differentiation. Extensive interference with
cellular growth control is exemplified by the cooperative action of v-erbA and its
retroviral partner v-erbB, both carried by the avian erythroblastosis virus (AEV),
which together induce acute erythroblastosis and fibrosarcomas in young chicks (Graf
and Beug, 1983). The v-erbB gene, a truncated version of the chicken epidermal
growth factor receptor, is necessary and sufficient for the transformation of chicken
embryo fibroblasts by AEV (Sealy *et al.*, 1983; Frykberg *et al.*, 1983). v-ErbB
initially induces proliferation of erythroid stem cells, but does not block their
differentiation program (Frykberg *et al.*, 1983). As a consequence, erythrocytic cells
transformed by viruses carrying only the v-erbB oncogene spontaneously differentiate
at a high rate and stop multiplying (Frykberg *et al.*, 1983; Yamamoto *et al.*, 1983).
v-erbA, acting in conjunction with v-erbB, is able to block differentiation and to
inhibit the expression of genes required for the mature phenotype (Fuerstenberg *et al.*, 1992; Schroeder *et al.*, 1990; reviewed in Privalsky, 1992; Damm, 1993). v-ErbA
arrests the stem cells transformed by v-ErbB at the CFU-E (colony-forming unit-
erthroid) stage (Samarut and Gazzolo, 1982; Beug *et al.*, 1982) and thereby
maintains their proliferative potential (Frykberg *et al.*, 1983). Other oncogenes
encoding cytoplasmic proteins, like v-src, v-fps, v-sea, and v-Ha-ras are able to
replace v-erbB by cooperating with v-erbA in erythroid transformation (Kahn et al., 1986).

As described in detail in Chapter II, the v-erbA product dominantly represses the actions of normal TR and RAR. While chicken embryo fibroblasts expressing only v-erbA do not display a fully transformed phenotype, they exhibit a greatly enhanced growth potential and a decreased requirement for growth factors (Gandrillon et al., 1987). These growth-promoting properties of v-ErbA have so far exclusively been linked to dominant repression of the anti-mitogenic roles of TR and RAR. This mode of action is indicated by the fact that v-erbA is able to stimulate proliferation by overcoming growth inhibition by retinoic acid (Sharif and Privalsky, 1991). In this context, v-erbA function is strongly correlated with its action as a dominant negative oncogene in abrogating AP-1 repression by RAR and TR (Desbois et al., 1991a, 1991b; Zhang et al., 1991). Furthermore, v-erbA renders erythrocytic progenitor cells insensitive to the induction of apoptosis, and of self-renewal inhibition, by retinoic acid and thyroid hormone (Gandrillon et al., 1994).

(2) Meiotic induction of *Xenopus laevis* oocytes by progesterone and oncogene products

Oncogene involvement in the signal transduction events of the cell cycle can be studied by maturation assays carried out in oocytes from *Xenopus laevis*. Fully grown oocytes are arrested at the G2/M border and can be induced to enter M-phase by progesterone, insulin, or a range of introduced oncogene products (reviewed in Smith, 1989; Daar et al., 1991; Graziani et al., 1992; Grieco et al., 1995). Within minutes of progesterone exposure, a decrease in levels of cyclic AMP (cAMP)
occurs, mediated by inhibition of adenylate cyclase, which in turn downregulates protein kinase A (PKA) and protein kinase C (PKC) activities (Smith, 1989; Matten et al., 1994). Following hormonal stimulation, de novo translation of c-mos kinase occurs (Sagata et al., 1988; Yew et al., 1992; Sheets et al., 1995) and a number of other cytoplasmic kinases are activated, including Raf-1 serine-threonine kinase (Fabian et al., 1993; Muslin et al., 1993b), phosphatidylinositol 3-kinase (Muslin et al., 1993a) and MAPK kinase (Mordret, 1993; Posada et al., 1993; Kosako et al., 1994a, 1994b). These phosphorylation cascades culminate in the activation of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK). MPF and MAPK trigger the events of maturation, including germinal vesicle breakdown (GVBD), chromosome condensation, and meiotic spindle formation, either directly or by activating other protein kinases (Bement and Capco, 1990; Smith, 1989). MPF contains the Xenopus homolog of the universal M-phase inducer p34\textsuperscript{cdc2}, a serine-threonine kinase with histone H1 phosphorylating activity, which is complexed with B-type cyclins (Gerhart et al., 1984; Maller, 1990; Minshull et al., 1991).

There is growing evidence for the existence of several complementary control pathways which regulate partially overlapping sets of events at the G2/M transition. In particular, early maturation events, occurring prior to chromosome condensation, can be induced in progesterone-treated oocytes in the absence of protein synthesis (Baltus et al., 1973; Drury and Schorderet-Slatkine, 1975); membrane vesicularization, lamin disassembly and chromosome condensation are independent processes (Newport and Spann, 1987; Bement and Capco, 1990); GVBD can occur in the absence of active CDC2 in mouse oocytes (Choi et al., 1991; Gavin et al., 1992,
1994); and GVBD and white spot formation in *Xenopus* oocytes can be biochemically separated (Muramatsu *et al.*, 1989; Waldmann *et al.*, 1990). In addition, GVBD in *Xenopus* oocytes and a pseudomitotic phenotype in HeLa cells are inducible by a *cdc2*-independent, NIMA-like pathway (Osmani *et al.*, 1991a, 1991b; Lu and Hunter, 1995).

(3) **Nuclear envelope dynamics during the cell cycle**

The nuclear membrane of eukaryotic cells separates the genome from the cytoplasm. This separation permits additional levels of regulation which are absent in prokaryotic cells, and thus constitutes a major landmark in evolution (Dingwall and Laskey, 1992). The nuclear membrane consists of two concentric lipid bilayers, which are perforated by nuclear pore complexes (NPCs) that serve as channels for molecular exchanges between the nucleus and cytoplasm. The inner nuclear membrane is lined by the nuclear lamina, composed of a network of lamin proteins, a specialized type of intermediate filament protein (reviewed in Dingwall and Laskey, 1992). At the beginning of M-phase in almost all eukaryotic cells, the nuclear DNA dissociates from the nuclear envelope and condenses to form compact chromosomes. In eukaryotic cells that undergo an open mitosis, the nuclear envelope vesicularizes and the nuclear lamins depolymerize. At the completion of mitosis, these membrane vesicles and lamin proteins bind to the surface of condensed DNA and reestablish an intact nuclear envelope (reviewed in Gerace and Foisner, 1994; Goldberg and Allen, 1995; Dingwall and Laskey, 1992). Of all the mitotic rearrangements that occur, the disassembly of the nuclear lamina is the best understood, whereas almost nothing is known about the mechanisms of membrane vesicularization and nuclear pore
The *Xenopus* oocyte nuclear membrane is an attractive model system for the investigation of dynamic interactions of its components during the passage through the cell cycle (reviewed in Gerace and Foisner, 1994). The nuclear membrane of the *Xenopus* oocyte possesses several specialized features, i.e., a high density of NPCs (Scheer *et al.*, 1976), and a nuclear lamina composed of a single lamin isomer (Benavente *et al.*, 1985; Stick and Hausen, 1985); moreover, the large size of the oocyte nuclei allows the manual isolation of nuclear membranes (Krohne and Franke, 1983). Cell-free systems, based on extracts from mature *Xenopus* eggs in either a mitotic or an interphase state, have allowed the elucidation of a number of processes involved in nuclear disassembly (see, for example, Lohka and Maller, 1985; Macaulay *et al.*, 1995; Miake-Lye and Kirschner, 1985; Newport and Spann, 1987; Pfaller *et al.*, 1991; Pfaller and Newport, 1995; reviewed in Gerace and Foisner, 1994) and nuclear reassembly respectively (see, for example, Lohka and Masui, 1984; Burke and Gerace, 1986; Newport, 1987; Pfaller *et al.*, 1991; Pfaller and Newport, 1995; reviewed in Almouzni and Wolffe, 1993b). Importantly, employing cell-free nuclear disassembly systems, it has been possible to dissociate GVBD from chromosome condensation (Newport and Spann, 1987). Lamina disassembly appears to be necessary but not sufficient for GVBD (Newport and Spann, 1987), and is controlled by reversible phosphorylation of lamin proteins (Miake-Lye and Kirschner, 1985; Burke and Gerace, 1986; Gerace and Blobel, 1980). MPF, the pivotal regulator of mitosis, is thought to play a direct role in the phosphorylation of the lamins at the onset of M-phase (Peter *et al.*, 1990). Finally, nuclear reassembly can be studied in *Xenopus* egg extracts. These extracts, which contain large stockpiles of
disassembled nuclear components, readily reconstitute nuclei around condensed chromosomes \textit{in vitro}; a process which occurs with astonishing spontaneity (Lohka and Masui, 1983; Burke and Gerace, 1986; Newport, 1987).

Annulate lamellae (AL), an additional intracellular membrane compartment, are found in the cytoplasm and consist of stacks of flattened membranes perforated by numerous and densely packed pore complexes (Kessel, 1992). AL are especially numerous in rapidly growing cells, such as male and female gametes, tumour cells and virally infected cells. AL are believed to store excess nuclear envelope and NPC components for later use, but might have additional functional roles (Kessel, 1992). AL disassemble, along with the oocyte nucleus, during meiotic maturation (Stafstrom and Staehelin, 1984; Bement and Capco, 1989; Kessel and Subtelny, 1981). During this process, pore complexes disappear and the membrane is transformed into distended cisternae (Kessel and Subtelny, 1981). The significance of these structural alterations remains unknown.

(4) \textit{Structure and function of nuclear pore complexes}

Nucleocytoplasmic transport plays a fundamental role in coordinating the functions of the nucleus and the cytoplasm (reviewed in Melchior and Gerace, 1995; Stochaj and Silver, 1992). Transport across the nuclear envelope is carried out by the NPCs which are believed to be similar in both structure and function in all eukaryotic organisms from yeast to mammals (reviewed in Forbes, 1992; Gerace, 1992; Hurt, 1993; Rout and Wente, 1994). The nuclear pore is a highly selective channel through which macromolecules must pass to enter or exit the nucleus (reviewed in Hinshaw, 1994). Most proteins are too large to diffuse through the NPCs and are
actively transported through gated channels in the NPCs by signal-mediated mechanisms (reviewed in Guiochon-Mantel and Milgrom, 1993; Melchior and Gerace, 1995; Stochaj and Silver, 1992). Mediated import of proteins into the nucleus is specified by short stretches of amino acids known as nuclear localization sequences (NLSs). NLSs do not possess a clear consensus sequence, but most NLSs are highly enriched in basic amino acids, and may be continuous, as in the simian virus 40 (SV40) large T antigen, or bipartite, as in nucleoplasm (Boulikas, 1994; LaCasse and Lefebvre, 1995; reviewed in Dingwall and Laskey, 1991). NLS-mediated nuclear import of proteins can be viewed as a two-step process, consisting of ATP-independent binding to the NPC, followed by ATP-dependent translocation through the pore channel (Görlich et al., 1994; Newmeyer et al., 1986a, 1986b; Newmeyer and Forbes, 1988; Richardson et al., 1988).

The NPC has dimensions of ~120 x 80 nm and a mass of ~125 x 10^6 D (Reichelt et al., 1990), and may contain up to 100 different polypeptides (Rout and Wente, 1994). Of these pore proteins, only a small number have been identified to date (reviewed in Rout and Wente, 1994; Doye and Hurt, 1995). Three-dimensional image reconstruction suggests that NPCs are comprised of nuclear and cytoplasmic rings, with eight spokes radiating toward a central transporter apparatus (Hinshaw et al., 1992; Hinshaw, 1994) (Fig. 16). A "central channel complex" is attached to the inside of the spokes and contains the gated channel involved in signal-mediated transport of macromolecules (Forbes, 1992; Hinshaw, 1994; Hinshaw et al., 1992; Melchior and Gerace, 1995). In addition, sets of fibrils emanate from both the nucleoplasmic and cytoplasmic rings (Forbes, 1992; Richardson et al., 1988; Ris and Malecki, 1993). In isolated nuclear envelopes of amphibian oocytes, the
Figure 16. Transverse thin section of a nuclear pore complex (NPC) in an isolated nuclear membrane from a stage V (Dumont, 1972) *Xenopus* oocyte. NM, nuclear membrane; CR, cytoplasmic ring; T, central transporter; CF, cytoplasmic fibrils; NF, nucleoplasmic fibrils. Bar: 50 nm.
nucleoplasmic fibrils, which are ~75-100 nm long, are joined at their distal ends in a basket-like structure (Jarnik and Aebi, 1991; Goldberg and Allen, 1992), whereas the structurally distinct cytoplasmic fibrils are <25-50 nm long and have no obvious distal connections (Forbes, 1992) (Fig. 16). The initial binding of NLS-bearing proteins, complexed with cytoplasmic transport factors, is thought to occur at the fibrils at the cytoplasmic rings of the NPCs (reviewed in Melchior and Gerace, 1995).

NPCs perform a crucial role in controlling cellular processes, and mutations in NPC proteins can cause nuclear transport defects, extensive changes in nuclear membrane structure, and abnormal nuclear migration in the yeast *Saccharomyces cerevisiae* (see, for example, Bogerd et al., 1994; for a recent review, see Doye and Hurt, 1995). Interestingly, certain viral proteins can modulate nucleocytoplasmic transport of mRNA. For example, the adenoviral E1B protein, bearing a NLS and localized at NPCs, inhibits export of host transcripts, while promoting export of viral mRNA (Liang et al., 1995). Other recent studies showed that the fibrils emanating from the cytoplasmic face of NPCs contain the proteins CAN/nup214 (Fornerod et al., 1995; Kraemer et al., 1994) and TPR/nup265 (Byrd et al., 1994), which have both been associated with oncogenesis in humans when fused to other genes. Thus, oncogenes may be capable of inducing oncogenesis by altering the functions of NPCs.
(5) **Summary of results**

To assess whether v-ErbA can interfere directly with cell cycle control pathways, I investigated the effects of expression of v-erbA in *Xenopus* oocytes. This study showed that v-ErbA initiates early to intermediate meiotic events in *Xenopus* oocytes. v-ErbA induced a rearrangement of pigment in the animal hemisphere resembling the meiotic white spot and extensive ultrastructural meiotic changes in the architecture of the nuclear membrane, annulate lamellae, and the nucleoli, while chromosome condensation and spindle formation did not occur. Meiotic induction by v-ErbA was not mimicked by a dominant negative *in vitro*-generated mutant of TR, suggesting that release from G₂ cell cycle arrest by v-ErbA was not mediated by dominant repression of endogenous TR. Furthermore, v-ErbA-initiated maturation was independent of the cAMP/MPF signal pathway, but required *de novo* gene expression. Interestingly, the v-erbA protein was partially localized to NPCs by immunogold labelling. A novel function is proposed for v-ErbA by which it might disrupt nuclear mitogenic controls.
2. **Materials and Methods**

(1) **Plasmids**

RS-\(v\)-erbA, RSh-TR\(B\) C122>A and pRSV-\(lac\)Z are described in Chapter II, Materials and Methods. The pKCR2-cea plasmid was a gift of B. Vennström (Karolinska Institute, Stockholm, Sweden), and contains the chicken \(c\)-erb\(A\) cDNA expressed from the SV40 promoter (Sap *et al.*, 1989). The pGEM-4Z vector was a gift of M. Privalsky (University of California, Davis, CA), and contains the \(gag\)-v-\(erb\)\(A\) cDNA under control of the SP6 promoter. The V\(3\) clone was a gift of A. Munoz (Instituto de Investigaciones Biomedicas, Madrid, Spain). The chimeric \(v\)/c-\(erb\)\(A\) clone V\(3\) contains the retroviral \(gag\) sequence in the \(Sac\)I-\(Dra\)II fragment of \(v\)-\(erb\)\(A\) joined to the subsequent \(Dra\)II-\(Apa\)I fragment of \(c\)-\(erb\)\(A\) under control of the phage T7 promoter (Munoz *et al.*, 1988).

(2) **Oocyte microinjections**

A lobe of ovary was surgically removed from an adult female *Xenopus laevis* and processed as described in Chapter II. Microinjections were performed according to published methods with modifications (see Chapter II). Defolliculated stage VI (Dumont, 1972) *Xenopus laevis* oocytes were microinjected with 5 ng \(v\)-\(erb\)\(A\) expression vector alone or with 2 ng pRSV-\(lac\)Z, or with 5 ng pRSV-\(lac\)Z as a control, in a fixed volume of 20 nl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) into the nucleus by the 'blind' injection method with the needle inserted in the center of the animal pole. Alternatively, 5 ng V\(3\) mRNA in a fixed volume of 50 nl TE, pH 7.6, was injected into the cytoplasm. In addition, internally labelled \(v\)-\(erb\)\(A\) protein...
was synthesized by Caroline F. Bunn from the pGEM-4Z template by *in vitro* coupled transcription/translation in rabbit reticulocyte lysate (Promega, Madison, WI) according to the manufacturer's specifications, using SP6 polymerase. Oocytes were incubated in cycloheximide (200 µg/ml) or actinomycin D (30 µg/ml) for 16 h, and subsequently microinjected into the cytoplasm with 50 nl *v-erbA* solution (approximately 100 pg *v-ErbA* in rabbit reticulocyte lysate). This procedure was also carried out by C. F. Bunn.

(3) *ELISA*

Thirty-six hours after microinjection of 2 ng pRSV-*lacZ* and 5 ng RS-*v-erbA*, healthy oocytes (uniform pigment, not mottled) were homogenized singly in 200 µl 0.25 M Tris buffer, pH 7.5, and centrifuged at 10,000 x g in a microfuge at 4°C for 10 min. The supernatant was then centrifuged as before. This extract was used in the determination of β-galactosidase protein expression levels by enzyme linked immunosorbertant assay (ELISA) according to the manufacturer's specifications (5 Prime-3 Prime, Boulder, CO), and as described in Chapter II. This assay was repeated two times with oocytes from different animals since there can be variability in transcriptional activity between different batches of oocytes (see Chapter II). For each assay, a standard curve utilizing four pure protein standards was prepared, to ensure that β-Gal concentrations of sample extracts fell within the linear range of the assay. The data on reporter gene expression levels were analyzed by nested analysis of variance (ANOVA) controlling for variability between females and between independent ELISA assays.
(4) **Incubation treatments and assessment of germinal vesicle movement and germinal vesicle breakdown**

Oocytes were incubated in (i) O-R2 medium (Allison et al. 1991) only; or (ii) progesterone (10 μg/ml) (Sigma, Chemical Co., St Louis, MO); (iii) taxol (10 μg/ml) (Sigma); (iv) forskolin (20 μM) (Sigma) and isobutylmethylxanthine (IBMX; 2 mM) (Sigma); (v) cycloheximide (200 μg/ml) (BDH Chemicals NZ Ltd., Palmerston North, New Zealand); or (vi) actinomycin D (30 μg/ml) (actinomycin CI; Boehringer Mannheim NZ Ltd., Auckland, New Zealand) in O-R2. Twenty-four hours after microinjection with 5 ng RS-v-erbA, pRSV-lacZ, or 50 nl v-erbA protein solution, and/or a 24-hour incubation treatment ([i]-[vi]), oocytes were scored for germinal vesicle movement (GVM, white spot) under a stereomicroscope. Oocytes were assessed for germinal vesicle breakdown (GVBD) by manual dissection after fixation in ice-cold 1% trichloroacetic acid (TCA) for 5 min. Oocytes were scored positive for GVBD when the nucleus could not be dissected intact due to nuclear instability.

(5) **Differential interference contrast microscopy**

Oocytes, incubated in progesterone, or microinjected with 5 ng RS-v-erbA, RSh-TRβ C122>A, or unrelated RSV-lacZ template, were fixed and embedded essentially as described by Hausen and Riebesell (1991). In brief, oocytes were fixed in Romeis fixative (25 ml saturated mercuric chloride, 20 ml 5% trichloroacetic acid, 15 ml 37% formaldehyde) for three hours. Subsequently, oocytes were dehydrated in an ethanol series of 50% (15 min), 70% (30 min), 80% (15 min), 90% (15 min), 95% (15 min), and 100% (3 x 10 min); then infiltrated in 50% glycol methacrylate infiltration solution (Polaron) in ethanol for 1 hour and left in 100% infiltration
solution overnight. Oocytes were embedded in glycol methacrylate (Polaron) in gelatine capsules under nitrogen at 37°C for 5 hours. Five micron sections were obtained with Ralph glass knives. In order to remove mercury precipitates, the dry sections were treated with an alcoholic iodine potassium iodide solution (2% iodine, 3% potassium iodide in 90% ethanol) for 2 min, washed in 0.25% sodium thiosulfate for 15 min, and thoroughly rinsed in distilled water. The sections were stained utilizing an azofuchsin/aniline blue/orange G triple staining method (Hausen and Riebesell, 1991). Three independent batches of oocytes were used, and 3 to 4 oocytes per treatment/per batch were analyzed using differential interference contrast microscopy. Oocyte sections were photographed using Agfa optima color film with a blue filter.

(6) Electron microscopy for ultrastructural analysis

In ultrastructural studies of *Xenopus* oocytes, long fixation times and special fixation procedures are necessary. Unless indicated otherwise, all procedures were carried out at 4°C. Oocytes were fixed in 2.5% glutaraldehyde and 0.05% low molecular weight tannic acid in O-R2 overnight. Oocytes were then washed in O-R2 three times for 30 min, and subsequently transferred to 2% OsO₄ (in ddH₂O) for 3 hours with one change. Oocytes were then washed in O-R2 for 30 min, and dehydrated in a graded ethanol series of 30% (30 min), 50% (30 min), 70% (overnight), 80% (20 min), 90% (20 min), 95% (15 min), and 100% (2 x 15 min). After the 70% ethanol step, all further procedures were carried out at room temperature. Oocytes were incubated in 100% acetone twice for 20 min, and then gradually embedded in Spurr's resin in acetone: 30% resin (1 hour), 50% resin
(3 hours), 70% resin (overnight), 90% resin (8 hours), and 100% resin (overnight).

Oocytes were transferred into 100% resin in molds and cured at 70°C for 24 hours. Ultra-thin sections (50 nm) were stained with 5% uranyl acetate in 50% ethanol for 20 min, washed in ddH₂O, stained with lead citrate for 20 min, and put through a final washing step in ddH₂O.

(7) Histone H1 kinase assay

Adult female *Xenopus laevis* were injected with 0.5 ml gonadotropin (from pregnant mares' serum, Sigma; 200 U/ml in sterile ddH₂O) subcutaneously into the dorsal lymph sac, to improve the synchrony of response to progesterone in oocytes. After four days, a lobe of ovary was surgically removed from the animal and processed as described above. Defolliculated stage VI (Dumont, 1972) oocytes were microinjected into the nucleus with 5 ng RS-v-erbA or incubated in progesterone (10 μg/ml). At specified times, oocytes extracts were prepared by homogenizing groups of 10 oocytes in 20 μl extraction buffer (20 mM Tris, pH 7.5, 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM Pefabloc [Boehringer Mannheim], and 1 mM dithiothreitol [DTT]). The homogenate was centrifuged at 10,000 x g for 10 min at 4°C to pellet yolk and pigment. Ten microliters of the supernatant were mixed and incubated for 20 min at 25°C with 10 μl kinase buffer (30 mM Tris, pH 7.5, 30 mM MgCl₂, 1 mCi/ml γ[^32P] ATP [Amersham], 3 mg/ml histone H1[Sigma]) (Kosako *et al.*, 1994b). The reaction was stopped by the addition of an equal volume of 2 X SDS sample buffer (4% SDS, 20% glycerol, 120 mM Tris, pH 6.8, 0.01% bromophenol blue) and boiling for 5
min. Samples were resolved by 12% SDS-polyacrylamide electrophoresis followed by autoradiography.

(8) **In vitro transcription of the V₃ construct**

The plasmid containing the chimeric v-/c-erbA gene V₃ under the control of the phage T7 promoter was linearized with Eco RI, extracted with phenol-chloroform and precipitated. Transcription was carried out with 1 µl T7 RNA polymerase (50 U/µl, Epicentre) in 4 µl 5X T7 RNA polymerase buffer (Epicentre), 2 µl DDT (100 mM), 1 µl RNASin (40 U/µl) (Promega), 0.6 µl of each of the four ribonucleotide triphosphates (100 mM), 3 µl 7mGpppG (20 mM) (New England Biolabs), and 1 µl template DNA at a concentration of 1 µg/µl, for 1h at 37°C. The mixture was then incubated with 1 µl of RNase-free DNase (10 U/µl) (Boehringer) in 9 µl DNase dilution buffer for 15 min at 37°C. The reaction was stopped by heating the mixture to 65°C, and the mRNA was precipitated with 2 µl 4 M LiCl and 60 µl of 100% ethanol, prechilled at -20°C, for 30 min at -80°C. The RNA was pelleted by centrifugation at 12,000 x g for 10 min, washed with cold 70% ethanol, dried under vacuum, and dissolved in 5 µl TE, pH 7.6.

(9) **Immunoprecipitation of oocyte fractions with an anti-v-erbA antibody**

Oocytes were microinjected with 5 ng RS-v-erbA, pKCR2-cea expression plasmid into the nucleus, or 5 ng V₃ mRNA into the cytoplasm, and incubated in a sterile microtitre plate (5 oocytes/well) in 30 µl of O-R2 with 1 mCi/ml L-[³⁵S] methionine (1000 Ci/mmol, Amersham Pty Ltd., Auckland, New Zealand) at 18°C for 24 h. Nuclear fractions of 10 or 20 pooled v-erbA-injected oocytes were prepared manually
as described in Chapter II, after fixation in 1% ice-cold TCA for 5 min. Samples were homogenized in 0.5 ml NET-2 (Allison et al., 1993) containing 0.1 mM PMSF. For preparation of nuclear membrane fractions, nuclei were manually isolated in nuclear isolation medium (NIM; 83 mM KCl, 17 mM NaCl, 10 mM Tris, pH 7.2, 5 mM Pefabloc) (Krohne and Franke, 1983) in a small siliconized petri dish, using watchmaker's forceps. Nuclei were freed from adhering yolk by aspirating them into a pipette with an inner bore diameter of 0.7-0.8 mm, then washed once in fresh NIM and transferred into nuclear envelope isolation medium (NEM; 83 mM KCl, 17 mM NaCl, 10 mM MgCl₂, 10 mM Tris, pH 7.2, 5 mM Pefabloc). Within 30-60 s, the nuclear content forms an opaque and compact aggregate from which the nuclear membrane (envelope) detaches. The nuclear membranes were isolated by aspirating nuclei into a pipette with an inner bore diameter of 0.3-0.4 mm, cleaned in fresh NEM by repeated aspiration into a small pipette and transferred into a microfuge tube. Ten or 20 nuclear envelopes were pooled per sample and collected by centrifugation for 4 min at 8,000 x g and resuspended by gentle aspiration in 0.5 ml NET-2 containing 5 mM Pefabloc.

For total soluble protein electrophoretic separations, 20 μl 2 X SDS sample buffer and 1 mM DTT were added to 20 μl homogenate from nuclear and nuclear membrane fractions, which were then resolved by 12% SDS-polyacrylamide gel electrophoresis and stained with Bio-Rad Silver Stain (Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand). Immunoprecipitations of the nuclear fraction and nuclear envelope samples were carried out as described in Chapter II, using 30 μl of monoclonal antibody LA038 against v-erbA residues 58-75.
(10) **Immunolabelling of isolated nuclear membranes**

Oocytes were microinjected with 5 ng RS-v-erbA or pKCR2-cea into the nucleus and incubated in O-R2 for 12 to 14 h. Nuclear membranes of 20 oocytes per sample were manually isolated as described above, and fixed in 2% formaldehyde, freshly made from paraformaldehyde in PBS (137 mM NaCl, 1.3 mM Na₂HPO₄, 2.5 mM NH₂PO₄, pH 7.2), for 10 min at 4°C. Immunolabelling with colloidal gold, employing a biotin-streptavidin bridging technique, was carried out following published procedures (Cordes et al., 1993) with modifications, and according to the manufacturer's specifications (Amersham). Membranes were collected by centrifugation at 8,000 x g for 4 min and washed three times in PBS, pH 7.2, containing 50 mM NH₄Cl. Membrane samples were then resuspended in 24 μl PBS, pH 7.2, with 6 μl anti-v-erbA antibody LA038, and incubated for 2 h at room temp. Membranes were then centrifuged at 8,000 x g for 4 min, and washed three times in PBS. Subsequently, membranes were resuspended in 99 μl PBS, pH 7.2, containing 1 μl biotinylated goat anti-mouse Ig antibody (Amersham), and incubated for 2 h at room temperature. The samples were then washed with PBS, pH 7.2, as described above. Next, membranes were incubated in 38 μl PBS, pH 7.2, with 2 μl AuroProbe EM streptavidin 10 nm (Amersham) for 2 h at room temperature, and washed in PBS as before. Membranes were fixed in 2.5% glutaraldehyde in PBS, pH 7.2, for 30 min at 4°C, washed three times in PBS, followed by fixation in 2% aqueous OsO₄ for 30 min at 4°C and several washes in ddH₂O. Membrane samples were dehydrated in a graded ethanol series, incubated in 100% acetone twice for 20 min, and embedded in Spurr's resin in acetone: 50% resin (1 h), 90% resin (overnight), and 100% resin (1 h). Membranes were then immersed in fresh resin and cured at 70°C for 14 h.
Ultra-thin sections (50 nm) were stained with 2% uranyl acetate in 50% ethanol for 10 min, washed in ddH$_2$O, stained with lead citrate for 5 min, and washed in ddH$_2$O.

3. Results

(1) $v$-ErbA induced GVM and GVBD in Xenopus oocytes, but did not activate histone H1 kinase activity

During meiosis, oocytes undergo unequal divisions leading to polar body formation. For this process to occur, the oocyte nucleus (germinal vesicle) situated near the center of the oocyte, must migrate towards the animal hemisphere cortex and pigment granules in the region are rearranged, producing a whitish circular spot (germinal vesicle migration, GVM) (Lessman, 1987; reviewed in Smith, 1989). At the end of progesterone-induced GVM and GVBD, the white spot represents the area where the meiotic spindle has formed (Brachet et al., 1970; reviewed in Smith, 1989; Bement and Capco, 1990). Thus, the meiotically mature egg can be distinguished from the oocyte by the formation of this white spot which becomes delineated by a dark ring of rearranged pigment.

I first tested the ability of $v$-ErbA to initiate maturation in Xenopus oocytes by comparing the rate of GVM and GVBD in progesterone-treated and $v$-erbA-injected oocytes obtained from the same females. I have shown previously that $v$-ErbA, synthesized from a microinjected gene template in Xenopus oocytes, is functional and acts as a dominant repressor of TR (Chapter II; Nagl et al., 1995). In the present series of experiments, stage VI oocytes (Dumont, 1972) were injected into the
nucleus with 5 ng of v-erbA expression plasmid and cultured in O-R2 for 24 h, or incubated in progesterone for the same time period. Microinjection of v-erbA expression plasmid resulted in GVM. After 24 h, pigment granules were rearranged at the animal hemisphere cortex, forming a distinct, translucent circular spot with the nucleus visible underneath (Fig. 17A). The v-ErbA-induced pigment rearrangement was highly similar to the meiotic white spot induced by progesterone (Fig. 17A, cf. Panels b and c), although occasionally pigment aggregation in the center of the white spot in v-erbA-injected oocytes was seen (Panel d). For an assessment of whether GVM in v-erbA-injected oocytes is an active process or a consequence of degenerative changes in the cytoskeleton, I compared GVM in v-erbA-expressing oocytes with acrylamide-induced GVM (Lessman and Kessel, 1992). At present, the mechanism for positioning the oocyte nucleus is unknown, but is believed to involve an extensive intermediate filament network (Lessman and Kessel, 1992).

Acrylamide, an intermediate filament inhibitor, induced GVM in 20.5% of control oocytes positioned with the animal pole uppermost, but only elicited GVM in 2.6% of controls positioned in the opposite orientation (Table I). In contrast, 56.8% of v-erbA-injected oocytes exhibited a distinct white spot when orientated with the animal pole uppermost, while 27.8% of v-erbA-injected oocytes showed this feature when incubated with the vegetal pole uppermost. The depigmented patch at the animal pole of oocytes incubated with acrylamide, resulting from the displacement of pigment granules by the nucleus (data not shown), was distinct from the white spot seen in v-erbA-expressing oocytes (Fig. 17A). GVM occurred in 41% of v-erbA-injected oocytes oriented at random, while the mean frequency of GVM in
Figure 17. v-ErbA induces GVM and GVBD in Xenopus oocytes, but not MPF histone H1 kinase activity or chromosome condensation. (A) v-ErbA induced pigment rearrangements similar to meiotic white spot formation. Stage VI (Dumont, 1972) oocytes were injected with 5 ng lacZ expression plasmid (Panel a), incubated in 10 µg/ml progesterone (Panel b), or injected with 5 ng v-erbA template (Panels c and d), followed by examination of the pigment arrangement after 24 h. (B) GVM and GVBD induced by v-ErbA were not inhibited by forskolin/IBMX or taxol. Stage VI oocytes were injected with 5 ng v-erbA template (V) or 5 ng lacZ expression vector (C) and incubated in O-R2 alone, or in 10 µg/ml progesterone (P). Alternatively, microinjected or progesterone-treated oocytes were incubated in 20 µM forskolin/2 mM IBMX (forskolin), or 10 µg/ml taxol. After 24 h, the percentage of oocytes displaying GVM was assessed by inspection of whole oocytes, and the frequency of GVBD induction was scored by manual dissection of nuclei in 1% TCA. Three independent experiments were performed (30-40 oocytes/treatment/experiment). The error bars indicate the SEMs. (C) Chromatin remained decondensed, as v-ErbA did not inhibit transcription of a lacZ reporter gene. Oocytes were microinjected with 2 ng lacZ reporter gene construct alone (reporter only), or together with 5 ng v-erbA template (plus v-ErbA), and incubated for 36 h. Relative lacZ gene transcription was expressed in arbitrary units based on measurements of β-galactosidase protein levels by ELISA. Two independent experiments with 5 oocytes per treatment were performed. The error bars indicate the SEMs (p < 0.39). (D) v-ErbA did not induce MPF histone H1 kinase activity. After microinjection of 5 ng v-erbA or pRSV-lacZ template as a control (C) (upper panel), or incubation in progesterone or O-R2 (C) (lower panel), oocytes were collected at the indicated times and MPF kinase activity was assayed using histone H1 as a substrate and γ[32P] ATP. Samples were resolved by 12% SDS-polyacrylamide electrophoresis followed by autoradiography. MPF histone H1 kinase activity was assayed in oocytes with (+) or without (-) a distinct white spot (GVM). A representative example of three independent experiments, using oocytes from different female Xenopus, is shown.
B

% COOCES

OR-2  forskolin  taxol

V  P  C  V  P  C  V  P  C

D

V-ERB A

PROGESTERONE

GVM  -  -  +  -  +  -  +  -
Table I. GVM in stage VI (Dumont, 1972) *Xenopus laevis* oocytes in the presence of v-ErbA or acrylamide*.

<table>
<thead>
<tr>
<th>pole uppermost</th>
<th>animal</th>
<th>vegetal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GVM</td>
<td></td>
</tr>
<tr>
<td>total # oocytes</td>
<td># %</td>
<td>total #</td>
</tr>
<tr>
<td></td>
<td></td>
<td>oocytes</td>
</tr>
<tr>
<td>v-ErbA</td>
<td>44</td>
<td>25</td>
</tr>
<tr>
<td>acrylamide</td>
<td>34</td>
<td>7</td>
</tr>
</tbody>
</table>

* Oocytes were microinjected with 5 ng v-erbA expression template or incubated in acrylamide (10 mM) and cultured for 24 h in the orientation indicated, before scoring for GVM.
progesterone-stimulated oocytes was 69% (Fig. 17B). Thus, although positioning the animal pole uppermost facilitated nuclear migration in \textit{v-erbA}-expressing oocytes, GVM was observed with both orientations to gravity, indicating that \textit{v-erbA}-induced GVM is a directed process.

Next, oocytes were assessed for GVBD, and were scored positive when the nucleus could not be dissected intact. In 67% of oocytes that had been injected with \textit{v-erbA} template, nuclei were unstable and could not be dissected intact 24 h after microinjection (Fig. 17B). In progesterone-treated oocytes, GVBD occurred with a mean frequency of 94% after 24 h (Fig. 17B). It has been noted previously that GVM or white spot formation are not always apparent during maturation (Smith, 1989; Bement and Capco, 1990). In the present study, GVBD could be observed in the absence of GVM both in progesterone-treated and \textit{v-erbA}-injected oocytes. Taken together, these findings suggested that expression of \textit{v-erbA} in oocytes caused nuclear changes, which strongly resembled the events associated with maturation.

Substantial evidence exists indicating that the decrease in cAMP which follows progesterone treatment is both necessary and sufficient for meiotic maturation. For example, treatment of oocytes with forskolin, an adenylate cyclase activator, or IBMX, an inhibitor of cAMP breakdown, completely inhibits meiotic maturation (reviewed in Smith, 1989; Bement and Capco, 1990). To test whether \textit{v-ErbA} activated the cAMP-regulated CDC2/MPF pathway, I incubated \textit{v-erbA}-injected oocytes in forskolin and IBMX. Forskolin and IBMX did not significantly affect GVBD in \textit{v-erbA}-injected oocytes and decreased GVM only moderately, while completely inhibiting progesterone-induced GVM and GVBD (Fig. 17B). This result indicates that the nuclear changes induced by \textit{v-ErbA} do not require inhibition of
adenylate cyclase and a decrease in cAMP levels.

During meiosis, the organization of microtubules undergoes dramatic changes and eventually tubulin is assembled into the spindle complex. Activated MPF and MAPK alter microtubule dynamics by inducing the phosphorylation of microtubule-associated proteins (MAPs) which causes a drastic decrease in microtubule length (Bement and Capco, 1990; Davis, 1993; Gotoh et al., 1991; Matsuda et al., 1992; Mordret, 1993). Formation of the meiotic spindle, as well as GVM, can be blocked by experimentally interfering with the cytoskeletal targets of MAPKs (reviewed in Davis, 1993; Jessus et al., 1987; Lessman, 1987). Taxol, a microtubule stabilizer, inhibited GVM and GVBD induced by progesterone, but had no significant effect on GVM and GVBD in v-erbA-injected oocytes (Fig. 17B). This suggests that the nuclear changes mediated by v-ErbA did not involve rearrangements of the microtubular network; however, this does not rule out a role for other components of the cytoskeleton, such as intermediate filaments (Lessman and Kessel, 1992).

Another characteristic of progesterone-induced maturation is chromosome condensation and the cessation of transcriptional activity (Bement and Capco, 1990). Transcription of an unrelated lacZ gene template was unaffected by the presence of v-ErbA 36 h after microinjection (Fig. 17C), indicating that gene transcription was not shut down and chromatin remained decondensed.

MPF kinase activation, the definitive parameter of MPF-mediated oocyte maturation, can be measured in vitro by means of kinase activity of oocyte extracts, using histone H1 as a substrate (Langan et al., 1989). I assayed H1 histone kinase activity in v-erbA-injected and progesterone-incubated oocytes in three independent experiments with oocytes obtained from different female Xenopus. The results of a
representative experiment, using oocytes from the same female, are shown in Fig. 17D. In extracts prepared from v-erbA-microinjected oocytes, H1 kinase was not activated over an 18 h period when compared with an extract from lacZ-injected controls assayed after 2 h (Fig. 17D). The slightly elevated levels of H1 histone kinase activity in samples assayed 4 h and 14 h after microinjection were interpreted as not significant as they were not reproduced in repeat assays. Moreover, similar fluctuations in H1 histone kinase activity were also observed in extracts of lacZ-injected controls assayed over time (data not shown). Compared with a control extract from oocytes cultured in O-R2, extracts from oocytes incubated in progesterone exhibited high levels of histone H1 kinase activity after 2 h (2 h, GVM -), approximately 6 h prior to the appearance of the white spot (8 h, GVM +) (Fig. 17D). Both in the presence and absence of GVM, strong H1 histone kinase activity was present in extracts from progesterone-incubated oocytes over the entire time interval assayed, except for the extract from oocytes lacking a meiotic spot after a 10 h incubation (10 h, GVM -). The low H1 histone kinase activity in this sample was presumably due to the failure of some of the pooled oocytes to undergo meiotic maturation. In summary, these experiments showed that GVM and GVBD in the presence of v-ErbA were not caused by the activation of the cAMP-regulated CDC2/MPF pathway.

(2) v-ErbA initiated changes in the nuclear membrane characteristic of early and intermediate meiotic events

Many studies have relied solely on inspection of whole oocytes and dissection of nuclei to assay for GVM and GVBD respectively. However, this approach does not
allow the distinction of complete maturation from arrested meiosis which is also accompanied by nuclear destabilization (Baltus et al., 1973; Steinert et al., 1974). To enable me to discriminate between these two outcomes in v-erbA-injected oocytes, I performed a detailed analysis by semi-thin sectioning and interference contrast microscopy. Oocytes injected with v-erbA expression template showed pronounced morphological changes in the vegetal half of the nuclear membrane which were progressive over time (Fig. 18). It should be noted here that v-erbA-injected oocytes appeared fully viable 24 to 36 h after microinjection, as determined by the presence of dark, unmottled pigmentation of the animal hemisphere surrounding the white spot (Fig. 17A), and by comparison with degenerative changes in oocytes of low viability under interference contrast (Fig. 19). Degeneration in these oocytes was marked by the appearance of large vacuoles in the nucleus and cortex. As shown in Fig. 20A, extensive invaginations of the basal nuclear membrane and lamina were visible in v-erbA-expressing oocytes 16 h after microinjection. At 36 h, the invaginations appeared as sealed off lacunae of cytoplasm separated by stretches of nuclear membrane, and a yolk-free area was visible in the cytoplasm below (Fig. 20B). At this stage, the projections of nuclear membrane into the nucleoplasm were not restricted to the vegetal half of the nucleus, but could be seen along its circumference. In progesterone-induced maturation, the rupture of the nucleus always occurs in the vegetal half (Baltus et al., 1973; Bement and Capco, 1990; Hausen and Riebesell, 1991). In contrast, in v-erbA-injected oocytes, breakdown of the vegetal half of the nuclear membrane was not observed over a 42 h period. Instead, the dynamic rearrangements of the nuclear membrane eventually resulted in membrane rupture at the apical pole after 42 h (Fig. 20C), and
Figure 18. Semi-thin section of a stage VI (Dumont, 1972) *Xenopus* oocyte 36 h after microinjection of 5 ng of v-erbA expression construct, viewed under differential interference contrast. N, nucleus; CYT, cytoplasm. Bar: 100 μm.
Figure 19. Degenerative changes in a stage V (Dumont, 1972) *Xenopus* oocyte of low viability incubated in O-R2 for 20 h. N, nucleus; CYT, cytoplasm; C, cortex. Bar: 100 µm.
subsequent cytolysis at approximately 48 h (data not shown). Apical membrane rupture was not observed in lacZ-injected controls, demonstrating that rupture in v-erbA-injected oocytes was not due to damage during the process of microinjection (Fig. 20E). Nuclear membrane rupture at the apex, associated with arrested meiotic maturation, has previously been shown to be independent of the site of oocyte microinjection (Baltus et al., 1973; Steinert et al., 1974).

For comparison, progesterone-treated oocytes exhibited large invaginations of the basal nuclear membrane during intermediate meiotic maturation (16 h incubation), and nucleoplasm leaked into the cytoplasmic space, thereby creating a large yolk-free zone (Fig. 20D). After 26 h, meiotic spindle formation had occurred (Fig. 21). These observations showed that the structural alterations in the nuclear membrane of v-erbA-expressing oocytes, present 36 h after microinjection, were comparable to the nuclear membrane changes taking place during the intermediate stage of meiosis (Fig. 22). However, the transformations of nuclear membrane architecture occurred more slowly in v-erbA-expressing than in progesterone-induced oocytes; and the reorganization of the nuclear envelope was arrested at this intermediate stage in v-erbA-expressing oocytes.

(3) v-ErbA initiated ultrastructural transformations in the nucleus and the cortical region

The marked changes in cell ultrastructure that occur during oocyte maturation have been well characterized (Bement and Capco, 1990). Thus, to further investigate the alterations of the nuclear membrane in v-erbA-expressing oocytes, I carried out ultrastructural analysis by EM. First, to ensure that any ultrastructural changes
Figure 20. The structural changes in semi-thin sections of nuclei of *v-erbA*-expressing oocytes are indicative of meiotic induction. (A) Invaginations of the basal nuclear membrane and lamina in *v-erbA*-expressing oocytes 16 h after microinjection. (B) Progressive v-ErbA-mediated structural changes along the circumference of the nuclear membrane, and yolk-free zone below the basal nuclear membrane, 36 h after microinjection. (C) Rupture of the apical nuclear membrane (NM) in *v-erbA*-expressing oocytes 42 h after microinjection. (D) Invaginations of the basal nuclear membrane and lamina, and yolk-free space, in progesterone-induced oocytes after 16 h. (E) Absence of nuclear membrane invaginations in *lacZ*-injected control oocytes 36 h after microinjection. Bars: 100 μm.
Figure 21. Meiotic spindle in a stage VI (Dumont, 1972) *Xenopus* oocyte incubated in progesterone for 26 h. MS, meiotic spindle. Bar: 20 μm.
Figure 22. Semi-thin sections of nuclear membranes of stage VI (Dumont, 1972) \textit{v-erbA}-expressing and progesterone-induced oocytes, viewed under high power. Basal nuclear membranes of (A) a \textit{v-erbA}-expressing oocyte 36 h after microinjection, and (B) an oocyte after a 16 h incubation in progesterone (10 \textmu g/ml). Bars: 20 \textmu m.
observed were not the result of the process of microinjection, I compared \textit{lacZ} template-injected oocytes with \textit{v-erbA}-injected oocytes. \textit{LacZ}-injected oocytes were identical in appearance to uninjected oocytes. The vegetal half of the nucleus was extensively folded, forming narrow cytoplasmic channels and large lobes extending into the nucleoplasm (Fig. 23A). Annulate lamellae (AL) were often seen in the cytoplasm, in close proximity to the nuclear membrane (Fig. 23A).

In contrast, in \textit{v-erbA}-expressing oocytes, the nuclear membrane was drastically altered, while having maintained complete continuity, in the basal region. \textit{v-ErbA} induced the formation of long stretches of adjoined double membranes and the transformation of AL into cisternae (Fig. 23B-E). Seen in cross-section, the two double membranes appeared tightly adjoined to each other, except for small islands of enclosed cytoplasmic material (Fig. 23D and E). A striking feature of this membrane rearrangement was the alignment of NPCs across the joined membranes (Fig. 23F). High magnification revealed that the cytoplasmic annuli of the NPCs were joined to their counterparts across membranes by electron-dense material. The joined nuclear membranes, when manually isolated from oocyte nuclei, were firmly attached to each other and resistant to separation during tissue processing (data not shown). Furthermore, clustering of NPCs, with adjacent portions of nuclear membrane depleted of NPCs, was seen in the basal regions of the nuclear envelope (Fig. 23G and H). In regions of NPC clustering, projections of nuclear membrane extended into the nucleoplasm. Concomitant with the altered nuclear membrane architecture, multiple layers of cisternae devoid of NPCs developed (Fig. 23I), which typically contain fibrillar material attached to the inner side of the membrane (Kessel and Subtelny, 1981). In addition, the nucleoli showed signs of meiotic
Figure 23. Ultrastructural meiotic changes in thin sections of nuclei of v-erbA-expressing oocytes. (A) The basal nuclear membrane (NM) of control oocytes injected with 5 ng lacZ template was highly folded and formed narrow channels of cytoplasm (CYT) and lobes extending into the nucleus (N). Annulate lamellae (AL) were visible close to the nuclear membrane. M, mitochondrion. (B to J) Thin sections of v-erbA-expressing oocytes. (B) and (C) Extensive regions of nuclear membrane were tightly adjoined to each other 36 h after microinjection (adjoined membranes, AM). AL had become transformed into cisternae (C). (D) and (E) Long stretches of adjoined membranes (short arrows) with aligned NPCs (arrowheads) separated off islands of cytoplasm (CI) containing mitochondria and other cytoplasmic material. (F) NPCs (arrowheads) were aligned across the adjoined membranes (short arrows) by electron-dense material (long arrow). (G) and (H) NPCs in the basal nuclear membrane were clustered (large arrowheads), with adjacent regions being depleted of NPCs (small arrowheads), 36 h after microinjection. (I) AL were transformed into cisternae (C) devoid of NPCs. (J) The nucleoli (Nu) were segregated into distinct fibrillar and granular regions. Bars: (A) 1 μm; (B) 1 μm; (C) 1 μm; (D) 1 μm; (E) 1 μm; (F) 200 nm; (G) 200 nm; (H) 500 nm; (I) 200 nm; (J) 1 μm.
transformation (Fig. 23J), i.e., the granular and fibrillar parts had become clearly segregated (Steinert et al., 1974).

The cortex of v-erbA-expressing oocytes exhibited a subset of meiotic changes, but did not develop the characteristics belonging to the late stage of maturation (Fig. 24A-C). Microvilli were present 36 h after microinjection of v-erbA template (Fig. 24A and C), while they were lacking in oocytes, undergoing hormone-induced maturation, after 16 h (Fig. 24D). Pigment was clustered at a distance from the plasma membrane in both v-erbA-expressing (Fig. 24B) and progesterone-induced oocytes (Fig. 24E), resulting in the formation of a discrete white spot at the animal pole. In contrast, the cortical granules of v-erbA-expressing oocytes remained predominantly localized adjacent to the plasma membrane in the area of the white spot (Fig. 24B), but were retracted into the oocyte interior in progesterone-treated oocytes (Fig. 24E and F). This finding is consistent with the observation that the rapid decrease in cAMP level triggered by progesterone correlates with the movement of cortical granules away from the plasma membrane, suggesting that cortical granule position is controlled by the level of intracellular cAMP (Bement and Capco, 1990). Since v-ErbA-induced changes occur without a decrease in cAMP, the cortical granules would be expected to remain at the plasma membrane. Although the movement of both pigment and cortical granules during maturation is thought to be mediated by an actin network in the oocyte cortex, cortical granules apparently do not become associated with the actin network until after GVBD is complete (reviewed in Bement and Capco, 1990). Our observations provide further evidence for differential control of localization and movement of the two types of granules. In oocytes microinjected with the lacZ plasmid, microvilli were more numerous and
Figure 24. The cortex of v-erbA-expressing oocytes showed a subset of meiotic transformations in thin and semi-thin sections. (A) In the cortex of v-erbA-expressing oocytes microvilli (MV) and yolk-free corridors (asterisk) were visible 36 h after microinjection. Cortical granules (CG) were localized immediately below the plasma membrane. EM, extracellular matrix; P, pigment granules; Y, yolk platelet. (B) Pigment granules were retracted and clustered in the cytoplasm, while cortical granules (arrowheads) were present adjacent to the apical plasma membrane, in the area of the white spot 36 h after microinjection of v-erbA (semi-thin section). (C) The microvilli of v-erbA-expressing oocytes were partially retracted and of low density. The cortical endoplasmic reticulum (CER) (arrowheads) had not formed an extensive network and did not encircle the cortical granules. (D) Progesterone-induced maturation resulted in the complete retraction of microvilli after 16 h; yolk-free corridors were disrupted, and mitochondria (M) were randomly dispersed throughout the cortex. (E) Progesterone induction caused the retraction of cortical granules and the clustering of pigment granules in the cytoplasm (semi-thin section). (F) Retraction of almost all cortical granules, pigment granules, and other vesicles, resulted in an area of clear cytoplasm in the meiotic white spot (MS) of progesterone-induced oocytes after 16 h. (G) Cortex of a lacZ-injected control oocyte 36 h after microinjection. (H) Continuous pigment layer adjacent to plasma membrane in lacZ-injected oocyte 36 h after microinjection (semi-thin section). (I) Numerous extended microvilli (MV) in lacZ-injected oocyte 36 h after microinjection. The CER (arrowheads) was comparable to that of v-erbA-injected oocytes. Bars: (A) 1 μm; (B) 100 μm; (C) 500 nm; (D) 1 μm; (E) 100 μm; (F) 1 μm; (G) 1 μm; (H) 100 μm; (I) 500 nm.
further extended (Fig. 24G and I), than in v-erbA-injected oocytes (Fig. 24A and C). The layer of pigment granules was continuous in lacZ-injected oocytes and located directly adjacent to the plasma membrane (Fig. 24H), differing markedly from the clustered pigment arrangement in v-erbA-injected oocytes (Fig. 24B). Finally, two characteristic features of late maturation, disruption of the yolk-free corridors and development of an extensive cortical endoplasmic reticulum, were not seen in v-erbA-expressing oocytes (Fig. 24A and C). The structure of the cortical endoplasmic reticulum in lacZ-injected controls (Fig. 24I) was comparable to that of the cortical endoplasmic reticulum in v-erbA-expressing oocytes (Fig. 24C). In conclusion, structural analysis provides convincing evidence that v-ErbA induces early to intermediate meiotic events in Xenopus oocytes. A summary of the comparison of ultrastructural changes induced by progesterone and v-ErbA is presented in Table II.
Table II. Induction of early and intermediate meiotic events by v-ErbA in stage VI (Dumont, 1972) *Xenopus* oocytes.

<table>
<thead>
<tr>
<th>Timing of meiotic events&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Inducer</th>
<th>v-ErbA&lt;sup&gt;2&lt;/sup&gt;</th>
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<tr>
<td><strong>early</strong></td>
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<td>AL transformation</td>
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<td>basal membrane changes</td>
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<tr>
<td>pigment rearrangement</td>
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<td>NPC repositioning</td>
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<td>nuclear migration</td>
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<tr>
<td>nucleolar dissolution</td>
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<td>microvilli retraction</td>
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<td>GVBD</td>
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<td>cortical ER development</td>
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<td>meiotic spindle</td>
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<sup>1</sup> Maturation events were identified according to Bement and Capco (1990); Brachet *et al.* (1970); Kessel and Subtelny (1981); Kessel (1992); and Steinert *et al.* (1974).

<sup>2</sup> The presence (+) or absence (-) of events, indicative of different stages of progesterone-induced maturation, in *v-erbA*-expressing oocytes is shown (see Figs. 23 and 24).

<sup>3</sup> Meiotic events were timed as described in Bement and Capco (1990).
Some of the growth-promoting properties of v-ErbA are mediated by dominant repression of TR and RAR (Desbois et al., 1991a, 1991b; Zhang et al., 1991; Gandrillon et al., 1994; Sharif and Privalsky, 1991). Stage VI Xenopus oocytes contain low levels of TRα and TRβ protein (Eliceiri and Brown, 1994), but lack thyroid hormone (T₃) (Chapter II; Nagl et al., 1995). Conceivably, unliganded TR might contribute to the G₂ arrest by repressing certain M-phase-inducing genes. Therefore, I wished to assess whether dominant negative inactivation of unliganded TR by v-ErbA was responsible for the induction of early meiotic events. I have shown in Chapter II that the action of endogenous oocyte TR can be blocked by a dominant negative in vitro-generated mutant of human TRβ (TR C122>A) (Nagl et al., 1995). This DNA-binding deficient mutant is thought to block TR-mediated gene regulation by forming inactive dimers with TR (Nelson et al., 1993). When v-ErbA was replaced by the mutant TR C122>A, the morphology of the basal nuclear membrane in TR C122>A-injected oocytes was identical to that of untreated G₂-arrested oocytes (Fig. 25A). This finding suggested that v-ErbA did not initiate maturation events by acting as a dominant antagonist of endogenous TR and pointed to a direct role in gene activation for v-ErbA.

Thus, to test whether release of the G₂ arrest by v-ErbA required de novo gene expression, the frequency of GVBD was scored in oocytes that had been injected with in vitro-synthesized v-erbA protein and incubated in actinomycin D, an inhibitor of transcription, or cycloheximide, an inhibitor of protein synthesis. This experiment was carried out in collaboration with Caroline F. Bunn. Since v-ErbA was not purified from the translation reaction mixture, it was possible that the rabbit
Figure 25. v-ErbA acts at the level of gene activation, but not as an antagonist of TR. (A) A dominant negative mutant of TR has no effect on nuclear structure. The basal nuclear membrane of a TR C122>A-injected oocyte 36 h after microinjection. Bar: 20 μm. (B) GVBD initiation by v-ErbA required de novo gene expression. The frequency of GVBD was scored by dissection of nuclei from uninjected controls, or from oocytes injected with in vitro-synthesized v-ErbA (in rabbit reticulocyte lysate), after a 24 h incubation in O-R2, 30 μg/ml actinomycin D (Act. D), or 200 μg/ml cycloheximide (CXD). As an additional control, oocytes were injected with the rabbit reticulocyte lysate transcription/translation mixture (TNT) and scored for GVBD. The error bars indicate the SEMs of 3 independent experiments (30-40 oocytes/treatment/experiment).
reticulocyte lysate could supply factors that would effect GVBD. Thus, oocytes were injected with lysate mixture as a control. As shown in Fig. 25B, the lysate alone did not induce GVBD. Twenty-four hours after microinjection, the mean frequency of GVBD in oocytes injected with v-ErbA, and incubated in the absence of inhibitors, was 61% (Fig. 25B). Incubation of v-ErbA-injected oocytes in either actinomycin D or cycloheximide significantly reduced the frequency of GVBD to 7.5% (Fig. 25B), showing that meiotic induction by v-ErbA is dependent on mRNA synthesis and translation. Taken together, these results suggest that v-ErbA initiates meiotic events by activating a key regulator gene(s) in oocytes.

(5) A portion of v-ErbA was present at nuclear pore complexes

Srp1, a *Saccharomyces cerevisiae* homolog of importin, the vertebrate nuclear localization sequence (NLS) receptor, is essential for nuclear protein import, and is required for mitosis (Loeb *et al.*, 1995). Conditional *srp1* mutants arrest in the cell cycle during the G2/M phase, suggesting that import of cell cycle regulators into the nucleus is critical for cell cycle progression. Thus, it was of interest to determine whether v-ErbA associated with NPCs, thereby potentially modulating nucleocytoplasmic transport of cell cycle regulators. In immunoprecipitation assays, the majority of v-erbA protein, expressed from the RS-v-erbA construct, is recovered from manually isolated oocyte nuclei, while approximately 10% of v-ErbA partitions to the cytoplasmic fraction (Chapter II). As shown in Fig. 26A, a protein with a molecular weight of approximately 75 kD, corresponding in size to the full-length gag-v-erbA fusion protein encoded by the RS-v-erbA expression template (Privalsky, 1992), was predominantly detected in total soluble protein fractions from nuclei 8 h
Figure 26. A portion of v-ErbA was present at NPCs. (A) Low amounts of v-ErbA were associated with the nuclear membrane. Panel a: Total soluble protein fractions were prepared from 10 manually isolated nuclei (N) or nuclear membranes (NM), 0 or 8 h after microinjection of 5 ng v-erbA expression template, and resolved by 12% SDS-PAGE and silver staining. The arrowhead indicates a protein of approximately 75 kD, corresponding in size to the full-length gag-v-erbA fusion protein. Molecular weights (D) are indicated by dashes: 85,200; 55,600; 39,200; 26,600; 20,100; 14,300. Panel b: Total soluble protein fractions from 10 nuclei or 10 nuclear membranes 24 h after microinjection. Symbols are as described in Panel a. Panel c: The 75 kD gag-v-erbA protein was immunoprecipitated from the nuclear membrane and nuclear fractions using anti-v/c-erbA antibodies. Oocytes microinjected with 5 ng v-erbA expression vector were cultured in O-R2 medium with 1 mCi/ml L-[35S] methionine for 24 h, and nuclear membranes and nuclei were manually isolated. Nuclear membrane (NM) and nuclear (N) fractions of 10 v-erbA-injected oocytes were incubated with protein G-Sepharose-antibody complexes in an immunoprecipitation assay. Labelled polypeptides were recovered and separated by 12% SDS-PAGE followed by fluorography. Panel d: c-ErbA was exclusively immunoprecipitated from the nuclear fraction. Immunoprecipitation of c-erbA protein from the nuclear and nuclear membrane fractions of 20 oocytes injected with 5 ng c-erbA expression vector was carried out as described in Panel c. (B) Immunogold EM detection of v-ErbA at NPCs. Manually isolated nuclear membranes from v-erbA-injected oocytes were immunolabelled with 10-nm colloidal gold, using a v-erbA-specific monoclonal antibody in a biotin-streptavidin bridging technique, prior to embedding and thin sectioning. Panel a: An en face view of the cytoplasmic side of the nuclear envelope. Arrowheads indicate examples of colloidal gold-labelled NPCs clearly revealed by this plane of sectioning. Panels b and c: Selected examples of transversely sectioned NPCs labelled by the anti-v-erbA antibody. Panels d and e: Representative examples of tangentially sectioned nuclear envelopes. The v-erbA-specific antibody exclusively labelled the cytoplasmic fibrils of NPCs. The cytoplasmic side of all cross-sectioned nuclear envelopes faces the top of the figure. The cytoplasmic side of the nuclear envelope often forms blebs (Panel e). Bars: Panel a, 200 nm; Panel b, 100 nm; Panels c-e, 50 nm.
after microinjection of the gene template *(Panel a).* Interestingly, low levels of a protein of comparable size were also detectable in total soluble protein fractions from nuclear membranes after 8 h *(Panel a).* After 24 h, v-ErbA accumulation in both the nuclear and nuclear membrane fraction was further increased *(Panel b).* In addition, v-ErbA was immunoprecipitated from both fractions by a monoclonal antibody with dual specificity for both \( v\text{-}erbA \) and chicken \( c\text{-}erbA \) *(Freake et al., 1988)* *(Panel c).* In contrast, deliberate overexposure of the fluorograph showed that wild-type chicken c-ErbA, expressed from injected \( c\text{-}erbA \) templates, was exclusively immunoprecipitated from the nuclear fraction *(Panel d).*

To identify the precise location of the \( v\text{-}erbA \) product, I performed immunogold labelling of manually isolated nuclear membranes *(Fig. 26B).* Nuclear membranes, isolated from \( v\text{-}erbA \)-expressing oocytes, were exclusively decorated with gold particles at the cytoplasmic face of NPCs *(Panels a to c)* and, more specifically, the \( v\text{-}erbA \) product was localized to the fibrils emanating from the cytoplasmic annuli *(Panels d and e).* A possible reason for the relatively low labelling density, which contrasted with the strong signal obtained by immunoprecipitation, is that the primary antibody was raised against a synthetic peptide of \( v\text{-}erbA \) (residues 58-75) within the DNA binding domain. Since the \( v/c\text{-}erbA \) NLS is separated from the DNA binding domain by only 12 amino acid residues *(Fig. 27)* *(Dingwall and Laskey, 1991; Lacasse and Lefebvre, 1995)*, antibody accessibility might have been reduced due to steric hindrance between the DNA binding domain and a NPC component(s) interacting with the v-ErbA NLS. In summary, immunogold localization of v-ErbA to the cytoplasmic fibrils of NPCs points to the possibility that this oncogenic protein might mediate some of its effects by interfering with NPC function.
Figure 27. Amino acid sequence of the epitope within the DNA binding domain (residues 58-75) and the nuclear localization signal of v-ErbA. v-ErbA residues 58-75 (indicated by a line), against which the monoclonal antibody LA038 was raised, are part of the DNA binding domain. The nuclear localization signal (NLS) (boxed) is separated from the DNA binding domain by only 12 amino acid residues.
Preliminary results suggest that the association with NPCs might be mediated by the gag protein

The v-erbA oncogene is expressed as a fusion product with the viral gag sequence (Privalsky, 1992). The results described in the previous section pointed to a possible involvement of gag in mediating the association of v-ErbA with NPCs, since it was shown that c-ErbA alone was not localized to the nuclear membrane. Interestingly, in preliminary experiments, a chimeric gag-c-erbA product (V₃), was also immunoprecipitated from isolated nuclear membranes (Fig. 28). This finding supports the notion that the gag portion of the gag-v-erbA product might be mediating association with NPCs. Since NLSs have been identified in the gag products of some retroviruses, such as human immunodeficiency virus 1 (HIV-1), and foamy viruses (Bukrinsky et al., 1993; Schliephake and Rethwilm, 1994), I analyzed the gag sequence of the gag-v-erbA fusion product for sequence homologies with known NLS motifs (LaCasse and Lefebvre, 1995; Siomi and Dreyfuss, 1995). However, as shown in Fig. 29, the AEV gag sequence contains only dispersed hydrophobic residues, and does not exhibit any previously identified NLS motif. Therefore, it seems unlikely that the observed gag-mediated association of the gag-v-erbA protein with NPCs was caused by Gag interactions with a NLS receptor. On the other hand, the retroviral Gag proteins have been termed "particle-making machines", since they direct the formation of virion-like particles through Gag-Gag interactions, even when expressed in the absence of all other virus-encoded components (reviewed in Wills and Craven, 1991). It is therefore possible that Gag-Gag interactions between several gag-v-erbA proteins, simultaneously docked at the same NPC, caused the aggregation of these proteins and thereby blocked their
Figure 28. Immunoprecipitation of the gag-c-erbA product (V₃) from nuclear and nuclear membrane fractions. Oocytes injected with 5 ng V₃ mRNA into the cytoplasm (V₃), or uninjected control oocytes (C), were incubated in O-R2 medium with 1 mCi/ml L-[³⁵S] methionine for 24 h, and nuclear membranes and nuclei were manually isolated. Nuclear membrane (NM) and nuclear (N) fractions of 20 oocytes per sample were prepared and processed as described in Fig. 26.
Figure 29. AEV gag amino acid sequence (GeneBank Accession Number M32090 Y00044). Single letter amino acid code is used and hydrophobic amino acids (lysine, K; arginine, R) are underlined. Nucleotide sequence conversion was carried out by DNASIS open reading frame display.

nuclear import. The putative gag-dependence of v-ErbA association with NPCs was not investigated further, since it lies outside the scope of this investigation. It does, however, raise the interesting question of whether the Gag portion of the gag-v-erbA fusion protein, although it is not involved in transcriptional regulation by v-ErbA (Sap et al., 1989), might nevertheless contribute to the function of this oncogenic protein by affecting its subcellular localization.

4. Discussion

A detailed understanding of the role of oncogenes in meiotic induction, and in cell cycle control in general, requires the dissection of the complex processes through which subsets of biochemical signal cascades result in distinct structural reorganizations of the cell. My study identified a new role for the retroviral v-erbA
protein in partially lifting the cell cycle arrest at the $G_2/M$ transition in *Xenopus* oocytes, and, moreover, characterized the distinct subset of structural transformations induced by v-ErbA.

(1) **v-ErbA induces a subset of meiotic events in Xenopus oocytes independently of CDC2/MPF kinase**

The progressive changes in nuclear structure during oocyte maturation can serve as markers for temporal subsets of meiotic transformations. In the present study, I showed that v-erbA-expressing *Xenopus* oocytes exhibited structural changes associated with a subset of meiotic events occurring prior to chromatin condensation. These changes showed remarkable similarity to progesterone-induced events, despite key differences in the mode of induction. Early and intermediate meiotic events were induced by v-ErbA independently of the cAMP-regulated CDC2/MPF pathway, and required gene transcription. In contrast, the induction of *Xenopus* oocyte maturation by progesterone does not require gene transcription, but is absolutely dependent on polyadenylation and translation of stored mRNA coding for the serine-threonine kinase Mos (Sagata *et al.*, 1988; Yew *et al.*, 1992; Sheets *et al.*, 1995).

The identification of a distinct subset of meiotic characteristics induced by v-ErbA is in agreement with other studies, reviewed in the introduction to this chapter, which have shown that meiotic events can be biochemically separated (Baltus *et al.*, 1973; Drury and Schorderet-Slatkine, 1975; Newport and Spann, 1987; Bement and Capco, 1990; Choi *et al.*, 1991; Gavin *et al.*, 1992, 1994; Muramatsu *et al.*, 1989; Waldmann *et al.*, 1990). In addition, NIMA kinase induces nuclear breakdown, pigment rearrangement and chromatin condensation in *Xenopus* oocytes, but not
spindle formation or nuclear migration, and does not activate CDC2/MPF or MAPK (Lu and Hunter, 1995). Furthermore, the membrane-bound guanine nucleotide-binding protein Ras can induce maturation and activation of MAPK by both Mos-dependent and Mos-independent pathways, and can bypass changes in intracellular cAMP levels (Smith, 1989, and refs. therein; Hattori et al., 1992; Pomerance et al., 1992; Shibuya et al., 1992; Nebreda et al., 1993). Recently, it has been shown that cAMP-dependent protein kinase A inhibition of meiosis acts at more than one site, demonstrating that MAPK and MPF activation are not necessarily interdependent events during maturation (Matten et al., 1994). Thus, the changes associated with M-phase induction can be grouped into partially overlapping sets of events which are regulated by several complementary control pathways.

(2) v-ErbA releases the G2 arrest in oocytes, but meiosis is blocked at an intermediate stage

The rearrangements of the basal nuclear membrane, NPC clustering, AL transformation into cisternae, and segregation of the granular and fibrillar components of nucleoli, which were all observed in v-erbA-expressing oocytes, represent the initiation of events leading to the disassembly of the nuclear membrane and NPCs during meiosis (Bement and Capco, 1990; Kessel and Subtelny, 1981; Brachet et al., 1970; Steinert et al., 1974). However, the reorganization of the nuclear membrane in v-erbA-injected oocytes did not proceed past this intermediate stage, chromosomes remained decondensed, and eventually the nuclear membrane ruptured at the apical pole. Thus, although nuclear breakdown occurred, the endpoint of the v-erbA-induced pathway was cytolysis, rather than meiotic spindle formation.
CDC2/MPF, either directly or through activation of other protein kinases, induces the disassembly of the nuclear lamina filaments and NPCs, and the vesicularization of the nuclear membrane (reviewed in Bement and Capco, 1990; Smith, 1989); and chromosome condensation and meiotic spindle formation are mediated by the reorganization of microtubules by CDC2/MPF and MAPK (Gotoh et al., 1991; Bement and Capco, 1990). The failure of these progressive events to occur in v-erbA-expressing oocytes was correlated with the fact that CDC2/MPF kinase was not activated, and that v-ErbA-mediated nuclear changes were not inhibited by taxol. This suggests that the nuclear changes did not involve rearrangements of the microtubular network and, by implication, occurred independently of a MPF/MAPK requirement. NIMA-related kinases, which are thought to be substrates of CDC2, are also involved in the regulation of chromosome condensation (Osmani et al., 1991a, 1991b; O'Connell et al., 1994; Lu and Hunter, 1995; Ye et al., 1995; reviewed in Fry and Nigg, 1995). Interestingly, lack of nimA during mitosis causes the proliferation of endomembranes in and around nuclei and fragmentation of the nuclear membrane in Aspergillus nidulans (Osmani et al., 1991a). My findings suggest that release of the G₂ arrest by v-ErbA allows meiotic reinitiation up to a point where signal transmission by activated MPF, MAPK, and possibly NIMA-related protein kinases, becomes essential for M-phase progression.

There was a striking match between the v-erbA-induced events and the characteristic features seen in the induction of early maturation by the simultaneous treatment of oocytes with progesterone and cycloheximide, or by injection of extract prepared from progesterone-treated oocytes (Baltus et al., 1973; Steinert et al., 1974; Smith, 1989). This subset of structural changes, termed pseudomaturation, is induced
in the absence of *de novo* synthesis of *c-mos* protein and MPF activation (Drury and Schorderet-Slatkine, 1975; Wasserman and Masui, 1975; Kobayashi *et al.*, 1991), and includes adjoined nuclear membranes, mottling of the pigment layer, and condensation of the fibrillar core of the nucleoli (Baltus *et al.*, 1973; Steinert *et al.*, 1974). Moreover, chromosome condensation is never observed in pseudomaturation, and the eventual apical rupture of the nuclear membrane results in cytolysis (Brachet *et al.*, 1970; Steinert *et al.*, 1974; Drury and Schorderet-Slatkine, 1975). Induction of early maturation by microinjected, *in vitro*-synthesized v-ErbA, which required mRNA synthesis and translation, and induction of pseudomaturation in the absence of protein synthesis, appear to constitute two alternate pathways capable of initiating an overlapping set of events.

In oocytes, M-phase induction by v-ErbA, like pseudomaturation, is ultimately a destructive process. The reason for this most likely lies in the absence of additional signals from activated protein kinases, essential for the completion of maturation. Once the G₂/M restriction point is passed, the maturing oocyte is committed to undergo events leading to GVBD and lacks the ability to reverse the meiotic pathway. Consequently, the initiated structural transformations eventually lead to the disintegration of the oocyte. This situation is wholly different from the context in which v-ErbA functions in somatic cells. Somatic cells constantly receive a wide range of extracellular signals conveyed by growth factors, which activate mitogenic kinases (reviewed in Weinberg, 1989a). Abnormal activation of a gene(s), encoding a cell cycle inducer, by v-ErbA can be expected to act in a complementary manner to these events and enhance certain mitogenic signals. As a result, v-ErbA apparently does not cause nuclear dissolution in somatic cells, but confers a reduced requirement
for growth factors and an enhanced growth potential (Gandrillon et al., 1987). These effects of v-ErbA are further discussed in Chapter IV.

(3) Growth-promoting properties of v-ErbA

The question of whether the growth-promoting properties of v-ErbA can be exclusively attributed to dominant repression of TR and RAR, or whether it also requires the activation of specific v-ErbA-responsive genes, remains unanswered to date. My study showed that the release of the cell cycle arrest at the G$_S$/M transition point by v-ErbA required de novo gene expression, and occurred independently of cAMP regulation and CDC2/MPF kinase activation. Furthermore, v-ErbA apparently does not act by dominantly inhibiting endogenous TR, since a dominant negative mutant of TR had no effect on cell ultrastructure. Therefore, the putative target gene(s) is unlikely to be regulated by TR in oocytes.

Importantly, the cellular forms of several viral oncogenes are found as signalling components of the MAPK cascade, which suggests that this pathway mediates somatic cell transformation (reviewed in Davis, 1993; Mordret, 1993; Mansour et al., 1994). Within the nucleus, signal transduction by MAPK activates a range of transcription factors by phosphorylation, including the products of the c-jun and c-fos proto-oncogenes which form the so-called AP-1 complex (Mordret, 1993; Davis, 1993; Avruch et al., 1994). Conceivably, v-ErbA action might mimic the function of a MAPK-activated transcription factor in oocytes. It is interesting to note in this context that another member of the steroid/thyroid receptor superfamily, the oestrogen receptor, was recently shown to be phosphorylated by MAPK in vitro (Kato et al., 1995). Thus, v-ErbA might initiate maturation events by activating a
key gene target(s) not normally expressed in the oocyte, which is capable of triggering the release from G2 arrest and inducing a specific subset of structural transformations.

The ability of a cell to overcome dependence on growth factors, either partially or completely, and to grow in an autonomous fashion, forms the basis of oncogenic transformation. Previously, v-ErbA was shown to confer partial growth factor autonomy by, firstly, rendering cells unresponsive to a range of anti-mitogenic controls; i.e., v-ErbA abolishes AP-1 repression, induction of apoptosis, and self-renewal inhibition mediated by TR and RAR in erythroid precursors (Desbois et al., 1991a and 1991b; Zhang et al., 1991; Sharif and Privalsky, 1991; Saatcioglu et al., 1993b; Gandrillon et al., 1994). Secondly, v-ErbA can activate an indirect autocrine pathway by inducing the platelet-derived growth factor B/c-sis gene in glial cells, again by exerting a regulatory role opposite to that of TR (Iglesias et al., 1995). My study suggests that v-ErbA, acting as an activator, can contribute to the attainment of growth factor autonomy by interference with mitogenic pathways distinct from those involving TR.

(4) A potential role for v-ErbA at nuclear pore complexes

A portion of v-ErbA expressed in oocytes was present at the nuclear membrane at the cytoplasmic face of NPC complexes, near the cytoplasmic annuli. Specifically, colloidal gold-labelled v-ErbA was seen at the cytoplasmic fibrils of the NPCs, possibly at fibril components involved in NLS-mediated ligand docking (Forbes, 1992; Görlich et al., 1994). NPCs perform a crucial role in controlling cellular processes. In the yeast S. cerevisiae, mutations in NPC proteins can cause nuclear
transport defects, extensive changes in nuclear membrane structure, and abnormal
nuclear migration (reviewed in Doye and Hurt, 1995). That components of the
import machinery can also interfere with cell cycle regulation is demonstrated by the
effects of a mutation in the S. cerevisiae NLS receptor, Srp1, that targets proteins to
the NPC (Loeb et al., 1995). A conditional srp1 mutant arrests cells at the G2/M
border, and was suggested to act by blocking the import of a critical cell cycle
regulator. Similarly, Pendulin (or OHO31), a Drosphila tumour suppressor gene
product with sequence homology to Srp1, exhibits cell-cycle dependent nuclear
localization and is required for normal cell proliferation (Küssel and Frasch, 1995;
Török et al., 1995). In the release from G2 arrest in oocytes, potential effects of v-
ErbA at NPCs would be expected to play an early inductive role, as the association
of v-ErbA with NPCs occurs well in advance of meiotic events. An isolated local
effect of v-ErbA on nuclear membrane structure appears unlikely, since the set of
ultrastructural changes induced by v-ErbA, including the observed nuclear membrane
alterations, was fully consistent with the pleiomorphic effects of meiotic induction.
While the significance of the presence of v-ErbA at NPCs remains to be determined,
it is interesting to consider the possibility that, in addition to its intranuclear function
in gene activation, v-ErbA might modulate nucleocytoplasmic transport of a key cell
cycle regulator(s).
IV.

Conclusion

Until recently, an almost exclusive emphasis on the mutational aspects of oncogenesis, based on retroviral model systems, has tended to relegate the cell as a whole to a secondary background role. Contrasting with this approach, expression of TR and v-ErbA in *Xenopus* oocytes has highlighted the importance of cell-specific molecular interactions in defining the properties of both transcription factors. In Chapter II, it was shown that both the cell-specific nuclear environment and protein-DNA interactions at enhancers determine whether these nuclear factors act as repressors or activators of gene transcription. Importantly, the dominant negative phenotype of v-ErbA was shown not to be universal, but to be dependent on interactions with other nuclear factors and the nature of a given DNA binding site. The importance of these findings for an understanding of growth-promotion by v-ErbA is demonstrated in Chapter III, where it was shown that v-ErbA is able to partially lift the G$_i$/M cell cycle arrest in fully grown oocytes. v-ErbA induced a subset of early to intermediate meiotic events by activating *de novo* gene expression, suggesting that v-ErbA is able to interfere with mitogenic signalling pathways.

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Abbreviations used in this chapter: DR4, direct repeat of TRE half sites separated by 4 nucleotides; LTR, long terminal repeat; NF-κB, nuclear factor-κB; RXR, retinoid X receptor; Sp1, specificity protein 1; TR, thyroid hormone receptor; TRE, thyroid hormone response element.
(1) The action of nuclear hormone receptors is dependent on molecular interactions

The function of nuclear hormone receptors is still understood predominantly in a context-independent way, as being determined by their three-dimensional structures, which are implicit in their primary amino acid sequences; and, at a more reductive level, the functional domains within receptors are seen as autonomous modules. However, recent studies characterizing the modulating roles of protein-protein (Chen and Evans, 1995; Forman et al., 1995b; Hörlein et al., 1995; Kurokawa et al., 1995; Lee et al., 1995a and 1995b) and protein-nucleic acid interactions (Kurokawa et al., 1995; Rastinejad et al., 1995; Sjöberg and Vennström, 1995; Toney et al., 1993), as well as reports on interdomain allosteric regulation (Durand et al., 1994; Kurokawa et al., 1994), indicate that this view inadequately reflects nuclear receptor action. The findings on TR and v-ErbA function reported in my dissertation, together with these studies, demonstrate a pressing need for an expanded conceptual framework on which to base nuclear receptor research. Hence, I propose a context-sensitive model of nuclear receptor action, which views receptor protein functions as plastic properties, requiring definition by molecular interactions.

To allow responsiveness to the complex requirements of development, differentiation and the cell cycle, eukaryotic genes are coupled via cellular signal transduction networks to their extra- and intra-cellular environments. Cancer represents a range of disorders in cellular signal processing, mediated by the abnormal function of regulatory network components. In both healthy and neoplastic cells, the information transmitted to the genome via multiple signal pathways is combined at DNA control sequences by complex assemblages of transcription factors,
and then transmitted to the general transcription machinery. Many different types of signals are processed in this way; for example, information about a cell's lineage, its interactions with other cells, the stage of the cell division cycle, and hormonal signals. Individual regulatory elements function as multifactorial switches, each being responsible for only a certain aspect of the regulation of a given gene. Consequently, the full regulatory scope of a gene is only achieved by the integrated action of all, or of changing sub-sets, of its regulatory elements. This is well illustrated by the action of TR at TR-regulated genes which are commonly controlled by complex patterns of different control elements. The chicken lysozyme gene, for example, is regulated by the combined activity of thyroid and steroid response elements, enhancer sequences and silencer elements, to the effect that an entire chromatin loop with extensive flanking parts on both sides of the transcribed region acts as a regulatory unit (Sippel and Renkawitz, 1989).

Another consequence of the modular composition of complex enhancers is that different combinations of binding sites generate different transcriptional patterns. Modulation of the mode of action of TR/RXR heterodimers by different TREs provides an interesting example. My study showed that mammalian TR/RXR heterodimers constitutively activated transcription from a palindromic TRE linked to a viral promoter in *Xenopus* oocytes. In contrast, *Xenopus* TR/RXR heterodimers act as efficient activators and repressors of the 1.6 kb promoter of the *Xenopus* TRβA gene, containing several TREs within extensive flanking regions (Machuca *et al.*, 1995), in the presence or absence of T₃ respectively (Wong and Shi, 1995). While species-specific interactions between the nuclear receptors and general transcription factors might have contributed to these differences, this comparison underscores the
importance of enhancer composition for the action of TR and RXR. The effects of allosteric interactions between RXR and TR, or RXR and the orphan receptor Nurrl, serve to further illustrate this point (Forman et al., 1995). Heterodimerization between TR and RXR, bound at two consensus half-sites in a DR4 or palindromic arrangement, results in the suppression of ligand-induced transcriptional activities of RXR. Interestingly, without binding directly to DNA, RXR can heterodimerize with a Nurrl monomer bound to a single half-site. As a result of this interaction, the constitutive activity of Nurrl is suppressed, and the complex becomes 9-cis retinoic acid dependent. As an additional example of binding site complexity, the human immunodeficiency virus type 1 (HIV-1) LTR is activated by TR binding to TREs contained within the viral NF-κB and Sp1 DNA motifs (Desai-Yajnik and Samuels, 1993). At the Sp1 element, the binding of TR and Sp1 is mutually exclusive, and direct interaction between the retroviral tat protein and TR is required for TR binding and transcriptional induction of the TRE within the Sp1 motif.

Where oligomeric protein complexes bind to the same control sequence, different combinations of proteins can have different functional activities, including DNA binding specificities (Diamond et al., 1990; Glass, 1994). The consequences can range from efficient activation to complete inhibition, and can change in response to environmental and differentiation signals. Thus, the diversity provided by dimerization, between nuclear hormone receptors (Mangelsdorf and Evans, 1992) and among different nuclear factor families (Karin et al., 1993), offers enormous scope for regulation. In addition, interactions with tissue-specific factors can have profound effects on receptor action. My investigation demonstrated that the v-erbA protein, which is generally classified as a dominant negative transcription factor in animal
cells, is converted into a transcriptional activator by factors present in nuclear extracts from anterior pituitary cells. It follows that, if a factor can act as an activator or repressor depending upon its interactions with other proteins, it is the balance of these proteins in the cell and their relative strengths of interaction that is of paramount importance. In conclusion, the combinatorial nature of the transcription process strongly supports a context-sensitive model of nuclear receptor action.

Understanding the complexities of how molecular interactions, involving normal and oncogenic nuclear receptors, change in response to environmental, proliferation and differentiation signals will be an exciting focus of future research.

(2)  **Signalling networks and cellular architecture are interdependent**

v-ErbA initiated early to intermediate meiotic events in *Xenopus* oocytes by activating *de novo* gene expression. The finding that v-ErbA initiated the release from $G_2$ arrest in oocytes, but did not induce advanced meiotic events, highlights the strictly cooperative nature of the *v-erbA* oncogene. In somatic cells, v-ErbA depends on the presence of an oncogenic protein kinase, such as *v-erbB* or *v-src*, for the achievement of a fully transformed phenotype (Kahn *et al.*, 1986; Frykberg *et al.*, 1983). These observations emphasize that the effects of the *v-erbA* oncogene are embedded within a complex cellular signalling network made up of mutually interdependent components. Mitogenic signalling cascades constitute sequences of biochemical reactions that directly link signals initiated by receptors at the plasma membrane to specific cytoskeletal and transcriptional targets for control of the cell cycle and differentiation (Davis, 1993; Mordret, 1993). In the nucleus, cytoplasmic signalling cascades are integrated with the hormonal signals conveyed by steroid and
thyroid hormone receptors by complex cross-talk mechanisms (Desbois et al., 1991a, 1991b; Diamond et al., 1990; Jonat et al., 1990; Karin et al., 1993; Schüle et al., 1990a, 1990b; Zhang et al., 1991). Signal amplification along the branching structure of network cascades leads to the activation of increasing numbers of targets at successive levels of signal transduction. Through this mechanism, the action of a single oncogenic receptor kinase at the cell surface, such as v-ErbB, can be sufficient to induce complete cellular transformation; and expression of tyrosine kinase oncogenes, such as tpr-met and RET, efficiently induces maturation in oocytes (Daar et al., 1991; Grieco et al., 1995). In contrast, the oncogenic transcription factor v-ErbA, acting at the most distal level of mitogenic cascades, may only be able to activate a subset of genes essential for cellular growth-promotion, and, in oocytes, only induces a subset of meiotic events.

The transformation of an oocyte into an egg provides a valuable example for the general requirement of a combination of intracellular signals for the induction of an integrated structural and functional response. Meiosis is orchestrated by subsets of biochemical signal cascades, which independently regulate distinct structural reorganizations, with the ultimate outcome of dramatically altering cell function (Bement and Capco, 1990). Thus, intracellular signalling could be seen as a system which processes the information transmitted by all pathways in a parallel distributed network. Similar models of biological information processing have been proposed to explain such diverse processes as brain function (reviewed in Rowe, 1995) and differentiation and development (Atlan and Koppel, 1990). Possibly, these models reflect certain universal principles of signal processing, operating at all organizational levels in biological systems. A cell receives a constant stream of signals both from
within the cell itself and from the surrounding environment, and information is most likely carried by the changing patterns and intensities of all signalling events combined. These patterns are created by the substrate specificity of regulatory kinases in mitogenic cascades, and the binding strengths of protein-protein and protein-nucleic acid interactions in nuclear receptor signalling. Importantly, the cell-specific expression of numerous network components can greatly alter the response to upstream and cross-talk signals.

Cells interpret biological information within three-dimensional networks in which structure and function are mutually dependent. Unfortunately, studies of cell structure have often been judged to be of only observational interest and of lesser scientific value than biochemical approaches. However, the study of nuclear architecture is now moving to the forefront of molecular biology, as its crucial role in gene regulation (see, for example, Blobel, 1985; Nigg, 1988; Bidwell et al., 1993; Cremer et al., 1993; Dworetzky et al., 1992), differentiation, growth control and neoplastic transformation has been recognized (reviewed in Brasch and Ochs, 1995; Penman, 1995; Pienta et al., 1989). It has also been proposed that structural information, derived from cell-cell contacts or cell shape, can be transmitted to the nuclear matrix by the cytoskeleton, and might influence gene transcription (Cook, 1989; Pienta et al., 1989).

Genetic instability, activation of oncogenes and deregulated signal transduction are direct causes of cellular transformation. It is important to remember that virtually every component of the cytoskeleton and the nuclear matrix can also be altered in cancer cells; and that alteration of cell structure is in fact the morphological hallmark of cancer diagnosis (Pienta et al., 1989, and refs. therein). It is therefore necessary to
define the relationship between abnormal signalling events induced by oncogene products, cell structure and genetic changes. The products of oncogenes and their targets can be pictured as sensitive nodes within integrated regulatory-structural networks, whose activity is capable of profoundly altering the global state of the cell. These considerations motivated me to take a combined biochemical and ultrastructural approach in my investigation of v-ErbA-initiated maturation events in *Xenopus* oocytes. M-phase initiation by v-ErbA through gene activation, shown by my study, exemplifies oncogene action through interference with mitogenic signalling networks. In addition, I identified an abnormal subcellular localization for v-ErbA, which was partially found associated with the pore complexes at the cytoplasmic face of the nuclear envelope. This finding poses the intriguing question of whether v-ErbA can also disrupt regulatory networks by interfering with cellular architecture, in particular, components of NPCs. In conclusion, my study as a whole contributes a range of new perspectives on the v-erbA oncogene and its action within the regulatory-structural networks of cells.

(3) **Network models of neoplastic cells will open up new avenues in cancer research**

Biological models change with time (Keller, 1995). In the 1950s and 1960s, cellular processes were explained in terms of biochemical pathways and the thermodynamic principles of reaction kinetics. The ability of a cell to establish metabolic pathways was believed to depend solely on the execution of a fixed genetic program (reviewed in Keller, 1995). During this period, studies of two DNA tumour viruses, polyomavirus and simian virus 40, belonging to the papovavirus
group, suggested that the addition of one or a few genes could dramatically alter cell behaviour (reviewed in Eckhart, 1989). This led to the realization that neoplastic changes could be analyzed genetically and biochemically, and marked the beginning of experimental oncology (reviewed in Varmus, 1989). Cancer came to be seen as a genetic disease. Starting from this premise, experimental oncology has succeeded in identifying viral and cellular oncogenes as well as tumour suppressor genes, and has established models of neoplastic transformation possessing extraordinary explanatory power (reviewed in Chapter I). Importantly, models of collaborating viral oncogenes, such as v-erbA and v-erbB, have led to the multistep hypothesis of carcinogenesis (reviewed in Weinberg, 1989b).

In the 1990s, through the discovery of allosteric control, the intricacies of transcriptional regulation and the complexities of the cell cycle, cells are increasingly coming to be seen as intricate networks of structures and information. We now know that genomes do not function as fixed programs, and that cells actively regulate their genes in response to their own internal state (reviewed in McKnight and Yamamoto, 1992) and a wide range of signals from their environment, including those conveyed by hormones (reviewed in Hill and Treisman, 1995). Steroid and thyroid hormones comprise the broadest class of gene regulatory agents known (Brasch and Ochs, 1995; Detera-Wadleigh and Fanning, 1994; Laudet et al., 1992). Their receptors, belonging to the diverse superfamily of nuclear receptors, play a central role in the regulatory networks that transmit signals, originating from the extracellular environment, to the genome through a complex sequence of molecular interactions. Together, these insights have important implications for cancer research. In the United States, for example, hormone-related cancers account for more than
20% of all newly diagnosed male and more than 40% of all newly diagnosed female malignancies (Henderson et al., 1993). Hormonal signals contribute to the causation and progression of several human cancers, including cancer of the breast, endometrium, prostate and ovaries. The interplay between hormonal signals, normal and oncogenic nuclear receptors, and additional activated oncogenes therefore constitutes an area of primary importance in cancer research. Furthermore, oncogene function needs to be understood within the context of the global state of structural and regulatory networks of neoplastic cell types. In the future, the development of network models of neoplastic cells, reflecting these complexities, will expand our understanding of the role of oncogenes in human cancer.
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Appendix 1

The results of the investigation described in Chapter II were published and a reprint of the publication is included in Appendix 1.

In consultation with my supervisor, Dr. Lizabeth A. Allison, I designed and carried out the study described in this publication. Colleen C. Nelson prepared the TR C122>A clone and some of the reporter gene constructs while she was a postdoctoral fellow in the laboratory of Paul J. Romaniuk.

S. Nagl
Constitutive Transactivation by the Thyroid Hormone Receptor and a Novel Pattern of Activity of its Oncogenic Homolog v-ErbA in Xenopus Oocytes

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In Xenopus oocytes, the rat thyroid hormone receptor α (rTRα), but not its oncogenic homolog v-ErbA, constitutively activated thyroid hormone (T₃)-responsive reporter genes at four positive thyroid hormone-responsive elements (TREs). At a subset of the positive TREs tested, the addition of T₃ resulted in a further enhancement of reporter gene activation. In contrast, both rTRα and v-ErbA functioned as constitutive activators when bound to the clone 122 TREs, which are induced by unliganded TR in mammalian cells. Therefore, the responses of the ligand-independent activation domains of TR and v-ErbA to cell-specific and TRE-mediated induction are not equivalent. Coexpression of the human retinoid X receptor α (hRXRα) enhanced both ligand-dependent and ligand-independent activation functions of rTRα and human TRβ (hTRβ) at a palindromic TRE (TREp). An endogenous TR activity mediated T₃ induction of TREp, while being repressed by an in vitro-generated dominant negative mutant of TR. T₃-mediated gene activation, by exogenous or endogenous TR, was repressed by v-ErbA at three positive TREs, but not at the TRE from the third intron of the rat GH gene (rGH₃TRE). Interestingly, preinjection of nuclear protein extract from anterior pituitary cells converted v-ErbA into a constitutive activator at rGH₃TRE. The pituitary-specific factor Pit-1/GHF-1 or hRXRα did not induce activation by v-ErbA at rGH₃TRE, suggesting that the dominant negative phenotype of v-ErbA can be abolished by direct or indirect interactions with other nuclear factors. (Molecular Endocrinology 9: 1522-1532, 1995)
At positively regulated TREs, addition of thyroid hormone (T₃) results in the activation of transcription, suggesting that the hormone triggers a conformational change in TR that induces the activator function (for a review, see Ref. 5). At negatively regulated TREs, TRs act as transcriptional repressors when bound to T₃ (for reviews, see Refs. 4 and 5). In addition, two novel TREs that mediate strong activation by unliganded TR were recently identified, whereas addition of T₃ reverses this response (9, 10). Although TRs are able to bind TREs as monomers and homodimers, heterodimerization with auxiliary nuclear factors, notably retinoid X receptors (RXRs), results in increased affinity for the response element in vitro and enhanced activation of gene expression in vivo (Ref. 20; for a review, see Ref. 21).

The retroviral v-erbA oncogene, a highly mutated version of chicken TRE, acts as a dominant repressor in mammalian and avian cells, blocking activation of gene expression by liganded TRs (Refs. 14 and 16; for a review, see Ref. 22). Mutations within the C terminus of the v-erbA protein appear primarily responsible for its conversion into a constitutive transcriptional repressor, as these mutations result in loss of the ability to bind thyroid hormone at high affinity (Refs. 14, 16, and 17; for a review, see Ref. 22). v-erbA has retained some binding ability, since, surprisingly, v-erbA acts as a hormone-regulated activator in S. cerevisiae (11, 23). Two mutations within the DNA-binding domain, and mutations in an N-terminal region outside of the DNA binding domain, alter the range of DNA target sequences the v-erbA protein can bind to relative to TR (Refs. 17, and 24-26; for a review, see Ref. 22).

Oocytes of the frog *Xenopus laevis* are an exceptionally efficient assay system to characterize the functional properties of nuclear hormone receptors. TR, expressed from cloned genes microinjected into oocytes, acts as a hormone-regulated activator in *S. cerevisiae* (11, 23). These findings exemplify the utility of *Xenopus* oocytes for the characterization of cell-specific actions of nuclear receptor domains.

### RESULTS

#### Both Constitutive and T₃-Mediated Gene Activation Functions of TRα and TRβ Are Induced in Xenopus Oocytes and Enhanced by RXR

We sought to establish whether *Xenopus* oocytes can be used for the reconstitution of nuclear receptor networks in vivo. In this context, we wished to ascertain whether oocytes provide a cellular background for nuclear hormone receptor action similar to either yeast or mammalian cells or whether they constitute a unique context. TR mRNA is present at all stages of oogenesis (28) and in fertilized eggs (29); however, at the onset of our study, TR protein had not been detected by immunoprecipitation or Western immunoblotting (29), suggesting that maternal transcripts are stored for use later in embryogenesis. Results obtained by enzyme-linked immunosorbent assay (ELISA) showed that oocytes contain no detectable endogenous T₃ (<0.1 nM). After incubation in 100 nM T₃, oocytes exhibited an intracellular hormone concentration of ~5 nM (data not shown), which falls within the physiological range for T₃ action. Thus, we presumed that *Xenopus* oocytes would be suitable for experiments investigating transcriptional regulation by exogenous TR and v-erbA protein at a range of diverse TREs, both with and without added T₃.

TREs consist of six-base pair consensus sequences, termed half-sites. These half-sites can be oriented as imperfect inverted, direct, or divergent repeats, separated by nucleotide gaps of variable size (for a review, see Ref. 4). Naturally occurring promoters often show mixtures of half-sites in differing orientations. We sought to reflect the natural diversity of TREs by employing four positively regulated response elements (TREs) that mediate strong activation by TR. Addition of thyroid hormone at high affinity (Refs. 14, 16, and 18) did not inhibit the ligand-independent activation function of TR at a palindromic TRE in oocytes, while both with and without added T₃.

In conclusion, these results show that oocytes constitute a viable environment for the study of nuclear receptor biology in a vertebrate. This system is well-suited for the characterization of transcriptional regulation by TRs and auxillary nuclear factors, notably RXRs, that define the natural context of gene expression.
elements possessing different half-site characteristics; i.e., the direct repeat TRE in the rat malic enzyme gene promoter (maTRE) (30), the inverted palindromic TRE in the promoter region of the chicken lysozyme gene (lysTRE) (13), rGH3TRE (31), a synthetic palindromic TRE (TREP) (14), and one complex response element which is strongly induced by unliganded TR and repressed by T3 in mammalian cells (clone 122 TRES) (10).

A rat TRα (rTRα) expression plasmid and a chloramphenicol acetyltransferase (CAT) reporter gene under the control of one of five different TRES were microinjected into the oocyte nucleus. Unliganded rTRα induced transcription of all five TRE-regulated CAT reporter genes (Fig. 1). Constitutive reporter gene induction by rTRα was strongest when under the control of lysTRE, TREP, or the clone 122 TRES. CAT reporter gene transcription under control of three positively regulated TRES (maTRE, lysTRE, TREP) was further enhanced by the addition of T3 but was unresponsive to hormone with rGH3TRE (Fig. 1). In contrast to results obtained in CV1 (monkey kidney) cells (10), addition of T3 did not significantly repress TR-mediated transcription regulated by the clone 122 TRES (Fig. 1). Reporter gene induction by TR was shown to be dependent on the presence of a TRE in oocytes, as expression levels of the mouse mammary tumor virus (ΔMTV)-CAT reporter gene lacking a TRE sequence were not induced by coinjection of rTRα, in the presence or absence of T3 (Fig. 1).

Unexpectedly, there was a weak T3-dependent induction of the thymidine kinase (tk)-TREp-CAT and TREP-ΔMTV-CAT reporter constructs in the absence of exogenous TR, while the ΔMTV promoter alone was not responsive to T3 (Fig. 1). Contrary to our original assumptions, these results suggested the presence of low levels of an endogenous TR-like activity in oocytes. A recent study has now reported that Xenopus oocytes contain both TRα and TRβ protein (32). The observed T3-mediated induction of a CAT reporter gene under control of TREP in the absence of exogenous TR indicates that these endogenous TRs can function as T3-regulated activators.

The β isoform of the receptor, hTRβ, also constitutively induced transcription at TREP, albeit to a lesser degree than rTRα (Fig. 2). Gene activation by hTRβ was not significantly enhanced in the presence of hormone at TREP. Moreover, to achieve detectable levels of reporter gene induction by hTRβ, a 5 times greater amount of the expression vector (RShTRβ) had to be microinjected compared with RS-rTRα. This increased requirement might have been due to differences in the expression of the two constructs in oocytes or to the decreased binding affinity of TRβ for palindromic TRES (11, 33).

RXRs have recently been shown to heterodimerize with TRs, enhancing TR binding to TRE elements and augmenting T3-mediated transcriptional activation (for
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Two years, oocytes were cultured in the presence or absence of 100 nM T3 after microinjection with 2 ng v-erbA expression plasmids into the oocyte nucleus, together with the TREP-tk-CAT reporter construct. As shown in Fig. 2, hRXRa enhanced constitutive reporter gene activation by both rTRα and hTRβ. Coexpression of hRXRa increased T3-independent and T3-mediated reporter gene activation by rTRα to the same level. The presence of hRXRa enhanced the constitutive activator function of hTRβ (2.5 ng expression vector microinjected) to levels seen with unliganded rTRα alone (0.5 ng expression vector). In addition, with hRXRa present, hTRβ activated the reporter gene in a T3-responsive manner to the same level as rTRα.

To summarize, both rTRα and hTRβ constitutively activated reporter gene transcription at five different TREs, while activation was further inducible by T3 at a subset of TREs. Furthermore, coexpression of hRXRa enhanced both ligand-independent and ligand-dependent activation by rTRα and hTRβ at TREP. An endogenous TR activity mediated weak T3 induction of TREP.

**Only Certain Naturally Occurring TREs Act as Control Elements for Repression by v-erbA in the presence or absence of T3**

In mammalian and avian cells, TR activities are blocked by v-ErbA acting as a dominant repressor at the level of the TRE. However, this function of v-ErbA is not universal but depends on cellular context (11, 26). Potent suppression of basal transcription of T3-regulated genes in the absence of TR and T3 has also been shown in mammalian cells (14, 18) and has been implicated in v-ErbA oncogenesis (17, 22, 23, 34). We were arbitrarily assigned the value 1. Two or three independent experiments (five oocytes per treatment) were performed for each TRE-CAT reporter gene construct, and the mean fold induction over basal transcription was calculated. The error bars indicate the SEM."
sought to establish the action of v-ErbA in Xenopus oocytes in two ways. The first series of experiments tested the competitive interaction between v-ErbA and rTRα at naturally occurring TREs, by determining the extent to which v-ErbA was able to dominantly repress TR-induced activation of the reporter gene constructs. The second set of experiments tested the ability of v-ErbA to suppress basal promoter activity of the reporter genes in the absence of TR and T₃.

We carried out a series of coinjection experiments to test the dominant repressor function of v-ErbA. In both the presence and absence of T₃, v-ErbA acted as a dominant repressor of rTRα-mediated CAT reporter gene transcription from lySTRE, ma1TRE, and TREp (Fig. 3A). Repression was most complete at TREp. The dominant activity of v-ErbA was dose-dependent, as a 5-fold excess of the v-erbA expression plasmid (Fig. 3A) produced an even greater repression of TR-mediated induction of the reporter genes than an equal ratio of v-erbA and TR templates (data not shown). In contrast, v-erbA did not repress rTRα-mediated transcription under the control of rGH3TRE (Fig. 3A). However, coexpression of the cofactor hFXRα reduced transcription in the presence of rTRα and v-ErbA at this TRE (Fig. 3A).

In mammalian cells, v-ErbA represses basal transcription of T₁₃-responsive genes in the absence of T₃ (14, 18). In the present study, v-erbA did not act as a repressor of basal gene transcription under control of four different positively regulated TREs in oocytes (Fig. 3A). Repression of basal transcription by v-ErbA would have been detectable by the CAT ELISA employed, since the linear range of the assay (tasted down to Aₐ₀ₐ 0.15) extended below the CAT protein levels observed due to basal promoter activity (Aₐ₀ₐ 0.23-0.3) (data not shown). The loss of this function by v-erbA parallels the lack of repression of basal transcription by unliganded exogenous TR in oocytes. Importantly, expression of v-erbA in oocytes abolishes its function as a repressor of basal promoter activity but does not interfere with its dominant repressor potential.

We showed that dominant repression by v-ErbA in the oocyte is a specific effect in the following ways. First, transcription levels of the ΔMTV-CAT reporter plasmid were unaffected by the presence of rTRα and-or v-ErbA with and without T₃, showing that gene regulation by rTRα and v-ErbA is dependent on the presence of a TRE (Fig. 3A). Second, T₃-dependent induction by rTRα of the ΔMTV-lySTRE-CAT reporter gene was unaffected by the coinjection of the lacZ expression vector in place of the v-erbA construct (Fig. 3B). Both in the presence and absence of the lacZ vector, T₃ enhanced reporter gene expression 1.5-fold over levels induced by unliganded rTRα (Fig. 3B). This control was performed at a 2-fold greater concentration of the lacZ vector (5 ng) compared with experiments using v-erbA construct (2.5 ng). Dominant repression by v-ErbA was thereby shown not to be caused by 'nonspecific squelching' (35); i.e. essential transcription factors are not simply being titrated by the presence of an excess of v-erbA expression template.

The v-erbA protein binds T₃ only at very low affinity, due to several mutations in the C-terminal domain (14, 16, 36, 37). Presumably, this lack of hormone binding would render v-ErbA highly resistant to undergo a ligand-induced conformational change. For this reason, we were interested to ascertain how v-ErbA acts at the clone 122 TREs in oocytes. In mammalian cells, T₃ inhibits the activator function and induces the repressor function of TR at this TRE (10). In Fig. 1 we showed that, in oocytes, TR constitutively activated transcription at the clone 122 TREs. We also observed constitutive trans-activation of the reporter gene construct by v-ErbA (Fig. 3C). This induction by v-ErbA was not significantly affected by T₃. The v-erbA protein was also unresponsive to T₃ at lysTRE in oocytes (Fig. 3C).

In the previous section we reported that there was a weak T₃-dependent induction of the TREp-CAT reporter gene in the absence of exogenous TR, suggesting the presence of low levels of T₃-regulated endogenous TR in oocytes. The T₃-dependent reporter gene induction by endogenous TR was dominantly repressed to the same level by v-ErbA and a dominant negative in vitro-generated mutant of hTRβ (TR C122 > A) (Fig. 4). This DNA-binding deficient mutant is thought to repress TR-mediated gene transcription by forming inactive dimers with TR (24). Therefore, dominant repression by TR C122 > A suggests that endogenous TR was able to dimerize with the exogenous mutant TRβ. The slight T₃ dependence of transcription in the presence of TR C122 > A was interpreted as not biologically significant because of high sample variability. Repression of the reporter gene by v-ErbA or TR C122 > A was dependent on the presence of a TRE. The lacZ reporter gene, which lacks a TRE sequence, was expressed at levels similar to those of the CAT reporter gene and was not repressed by either v-ErbA or TR C122 > A (Fig. 4). In summary, our results show that v-ErbA displays an altered pattern of activity in oocytes, compared with both mammalian cells and the yeast, S. cerevisiae. v-ErbA did not repress basal reporter gene transcription yet acted as a dominant repressor of TR action at the positively regulated TREs tested, with the exception of rGH3TRE. At the clone 122 TREs, v-ErbA acted as a constitutive activator.

Ptuitary-Specific Transcriptional Regulation of rGH₃TRE by rTRα and v-ErbA

In Fig. 1 we showed that the T₃-dependent activator function of rTRα was not induced at rGH₃TRE in the presence of hormone. Previous studies have shown that optimal transcription of the rat GH gene (rGH) under control of TRE sequences in the promoter region is dependent on a cell-specific factor, Pit-1/ GHF-1, binding at upstream elements (38). Since the
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rGH gene is expressed only in somatotrophic cells of the anterior pituitary gland, gene regulation at rGH3TRE, the TRE from the third intron, presumably also requires cell-specific auxiliary factors of known or unknown origin. For this reason, we sought to reconstitute the nuclear environment of anterior pituitary cells in oocytes. Xenopus oocytes possess the experimentally attractive feature of correctly targeting nuclear proteins, injected into the oocyte cytoplasm, to the nucleus (see, for example, Ref. 39). In this experiment, nuclear protein extract from ovine anterior pituitary cells was injected into the oocyte cytoplasm 5 h before nuclear microinjection of gene templates.

When oocytes were microinjected with nuclear protein extract, both the hormone-independent and hormone-dependent activator functions of rTRα were induced at rGH3TRE (Fig. 5A). Surprisingly, under the same conditions, v-ErbA served as a constitutive activator at rGH3TRE, whose strength of activation exceeded that of rTRα (Fig. 5A). Increasing the amount of v-erbA expression template injected did not enhance this response further (data not shown). In contrast, v-ErbA did not affect basal promoter activity of this TRE-reporter construct in the absence of nuclear protein extract (Fig. 5A).

These findings suggested that auxiliary factor(s), present in the injected nuclear protein extract from anterior pituitary cells, significantly altered the effect of both rTRα and v-ErbA on reporter gene regulation at rGH3TRE in oocytes. Two plausible candidates for this auxiliary role are the pituitary-specific transcription factor Pit-1/GHF-1 and RXRα. To test this possibility, expression vectors for either Pit-1/GHF-1 or hRXRα were coinjected with rTRα or v-erbA constructs, and reporter gene transcription regulated by rGH3TRE was assayed. Transcription was weakly induced by hRXRα alone, while Pit-1/GHF-1 had no significant effect on basal promoter activity (Fig. 5B). The activity patterns of rTRα and v-ErbA observed in the presence of nuclear protein extract of anterior pituitary cells were not replicated in the presence of hRXRα or Pit-1/GHF-1 (Fig. 5B). Instead, coexpression of hRXRα or Pit-1, together with rTRα, reduced trans-activation of rGH3TRE (Fig. 5B) compared with levels of trans-activation by rTRα alone (Figs. 3A and 5A). Pit-1 repressed induction of rGH3TRE by liganded rTRα below basal transcription (Fig. 5B). Compared with levels in the presence of v-ErbA only (Fig. 5A), coexpression of hRXRα with v-ErbA induced transcription slightly, while Pit-1 had no effect (Fig. 5B). In the presence of hRXRα, transcription was induced equally by rTRα or v-ErbA. These effects were not further investigated, since this series of experiments was carried out to test hRXRα and Pit-1 as potential candidates for conferring
positive T₃-responsiveness on rTRα, and strong constitutive activator function on v-ErbA, at rGH₃TRE.

Taken together, these results suggest that auxiliary factor(s), other than RXR and Pit-1/GHF-1, present in nuclear protein extracts of anterior pituitary cells, are necessary for T₃ induction of rGH₃TRE by rTRα and conversion of v-ErbA into a constitutive activator at this TRE.

**DISCUSSION**

Both α- and β- Isoforms of TR Act as Constitutive Activators in Oocytes

In Xenopus oocytes, rTRα acted as a ligand-independent transcriptional activator at four positively regulated TREs (lysTRE, malTRE, TREP, and rGH₃TRE). With the exception of rGH₃TRE, addition of T₃ led to a further increase in CAT reporter gene expression by rTRα. The 3.4- to 7-fold T₃-dependent reporter gene induction observed in oocytes was comparable to activation in mammalian cells (13, 14, 40). Human TRβ also acted as a weak hormone-independent activator at TREP but was not further inducible by hormone. The observed differences in ligand-independent activation function between rTRα and hTRβ are most likely due to structural divergence in the N-terminal domain (6).

Both ligand-independent and ligand-induced activation functions (7-10, 41) exhibit distinct cell type and promoter context specificity in TR and other receptors belonging to the steroid/thyroid hormone receptor gene superfamily (Ref. 11; for further references, see Ref. 6). Constitutive trans-activation by TR has also been shown in the yeast, S. cerevisiae (11, 26), and in vitro transcription assays using extracts from NT2 embryonal carcinoma cells or Namalwa B cells (42). Taken together with these results, the ligand-independent activation function of TR in oocytes points to a possible requirement of inhibitory auxiliary factor(s) for transcriptional repression. The finding that gene activation by unliganded TR at the clone 122 TREs was not reversed by T₃ in oocytes, while being suppressed by ligand in CV1 cells (10), also points to a requirement for an inhibitory auxiliary factor for the suppression of trans-activation by TR. We have shown that hRXRα enhances both ligand-independent and ligand-induced activation functions of rTRα and hTRβ at TREP, in analogy to the enhancement of both activation functions of hTRβ by hRXRα seen in the yeast (43). Thus, RXRα is not the inhibitory factor in question. Our results are in close agreement with recent evidence suggesting that an inhibitory factor sup-

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**Fig. 5.** Pituitary-Specific Transcriptional Regulation of rGH₃TRE by rTRα and v-ErbA

**A.** Action of rTRα and v-ErbA at rGH₃TRE in the presence of pituitary nuclear extract from ovine anterior pituitary cells. Oocytes were either microinjected with 20 nl nuclear protein extract into the cytoplasm 5 h before nuclear injection of gene templates (+PNE) or microinjected with the gene templates in the absence of nuclear protein extract (−PNE). Oocytes were then cultured in the absence or presence of 100 nM T₃ (−T₃, +T₃) after microinjection with 2 ng ΔMTV-rGH₃TRE-CAT reporter gene construct alone (reporter only), or together with 0.5 ng rTRα (plus TR) or v-erbA expression vector (plus v-erbA). After 24 h, oocytes were assayed individually for CAT protein by ELISA. Basal transcription rates of the CAT reporter construct in the absence of cofactors and T₃ (reporter only, −PNE, −T₃) were arbitraril

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In both panels A and B, three independent experiments (five oocytes per treatment) were performed, and the mean fold induction over basal transcription was calculated. The error bars indicate the s.e.m.
presses trans-activation by unliganded TR in mamma­lian cells (19, 44) and which also showed that RXR was not the major inhibitory factor in two cell lines tested (44).

The v-erbA Protein Displays a Novel Pattern of Activity in Oocytes

Conversion of TR and v-erbA into ligand-independent transcriptional activators has been shown to be mediated either by cell-specific protein-protein interactions (8, 11) or by direct DNA binding (9, 10). A constitutive activation function has also been identified in the N-terminal region of v-erbA (9). Under conditions that induce the constitutive activation function of TR, v-erbA also acts as a constitutive activator in yeast (11) and in mammalian cells (9). In contrast, we show here that the oocyte-specific cellular background induces the ligand-independent activation function of rTRα, but not of v-erbA, at four positive TREs, suggesting that induction of this function can be differentially controlled in TRα and v-erbA within the same cellular context. On the other hand, both rTRα and v-erbA constitutively activated reporter gene transcription at the complex clone 122 TREs (Fig. 6). This TRE belongs to the same class of response elements as the TRE found in the Rous sarcoma virus (RSV)-long terminal repeat (LTR), which mediates strong T3-dependent activation function of rGH3TRE, but not the major inhibitory factor in two cellular context.

Binding to this type of TRE induces a conformational change and renders the constitutive N-terminal activation domain accessible to the transcription machinery (9). In summary, expression of TR and v-erbA in oocytes induced the constitutive activation function only in TR at positive TREs, whereas this function was activated in both TR and v-erbA at the clone 122 TREs in the same cellular context. It follows that the responses of the ligand-independent activation domains of TR and v-erbA to cell-specific and TRE-mediated induction are not equivalent.

In oocytes, v-erbA did not repress basal promoter activity under control of all four positive TREs tested (TREp, lystRE, maTRE, and rGH3TRE). Like TR, the v-erbA protein possesses a repressor function located in the hinge domain (18). Transcriptional repression by TR is observed on minimal promoters containing a TATA box and TRE sequence (12, 13), and cell-free transcription assays have shown that unliganded TR functions as an active repressor of T3-responsive genes by inhibiting the formation of a functional preinitiation complex (15). Presumably, repression by TR and v-erbA occurs by a similar mechanism, namely, by interference with general transcription factors. In oocytes, protein-protein interactions between the repressor domain of v-erbA and the general transcription machinery might be prevented by a lack of auxiliary inhibitory factor(s) or cell-specific characteristics of the general transcription complex.

Dominant Negative Repression by v-erbA Depends on the Nature of the TRE

The action of v-erbA at rGH3TRE has not been tested in mammalian cells to our knowledge. In oocytes, v-erbA failed to repress TR-mediated induction at rGH3TRE. At the same time, v-erbA dominantly repressed trans-activation by TR at three other positive TREs with diverse half-site characteristics (maTRE, lystRE, and TREp). The TRE-modulated action of v-erbA in oocytes is summarized in Fig. 6. Although both primary half-sites of rGH3TRE possess a favorable thymidine nucleotide at the fourth position (25, 31), and the sequence of the major binding site is identical to the AGGTGC/TA consensus motif (Fig. 6), it appears that v-erbA does not successfully compete with rTRα for binding due to the structure of rGH3TRE. As shown by other workers, heterodimerization with RXR-related factors can be essential for the dominant negative action of v-erbA (40, 45). In the present study, a reduced level of trans-activation of rGH3TRE by rTRα and v-erbA was observed in the presence of hRXRα. However, the significance of this finding is presently unclear, since hRXRα also reduced trans-activation by rTRα alone at this TRE.

An Activity in Nuclear Protein Extracts from Anterior Pituitary Cells Confers T3 Responsiveness to rTRα and Converted v-erbA into a Constitutive Activator at rGH3TRE

Our findings suggest that T3 induction of rGH3TRE is regulated in a cell-specific manner. In the presence of nuclear protein extract from anterior pituitary cells, the T3-dependent activation function of rTRα was induced at rGH3TRE in addition to T3-independent trans-activation by rTRα without extract. We showed that T3 responsiveness of rTRα was not mediated by hRXRα.

- **Fig. 6. Half-Site Sequences of TREs**

The identity of the nucleotide residue at the fourth position of the two primary half-sites in rGH3TRE is indicated (boxed italicized letter) (25). Intervening nucleotides between half-sites are indicated by numbers. Arrows indicate orientation of half-sites. Differential levels of dominant repression are expressed in relative terms; (+ +) indicates complete repression, (+1 +) indicates moderate repression, and (+) indicates weak repression by v-erbA.
or the pituitary-specific transcription factor Pit-1/GHF-1, which is required for efficient T3-induction of the GH gene in somatotrophs (38). General mammalian-type transcription factors might have been involved in conferring T3 responsivity to rTRα, since rGH-TRE is also T3-inducible in CV1 cells (31). The tripartite rGH-TRE, although located in the third intron of the rGH gene, exhibits a markedly higher affinity for TR, as well as greater gene induction ability, than the TRE located in the promoter region of the gene, indicating an important in vivo role for this TRE sequence (31). Further indications of the importance of regulatory regions in introns are the presence of a glucocorticoid-responsive element in the first intron of the human GH gene (46) and the requirement for the presence of introns in the rat GH gene for efficient expression in transgenic animals (47).

Interestingly, v-ErbA acted as an efficient constitutive activator at rGH-TRE in the presence of nuclear protein extract from anterior pituitary cells, whereas it had no effect on basal promoter activity under the control of rGH-TRE without extract. Coexpression of Pit-1-GHF-1 or hRXRa did not replicate the mode of action of the v-erbA protein as a constitutive activator seen with the nuclear protein extract. This finding suggests that indirect or direct interactions with an as yet unidentified nuclear factor present in pituitary cells result in a conformational change in v-ErbA at rGH-TRE that induces its constitutive activation function. The conversion of v-ErbA into a constitutive activator by nuclear protein extract from anterior pituitary cells at rGH-TRE suggests that the dominant negative phenotype of the v-erbA oncogene can be abolished by either direct or indirect interactions with other nuclear factors.

Our experimental approach illustrates how studies of exogenous receptors in oocytes, in conjunction with microinjected auxiliary proteins, may contribute to the characterization of cell-specific and TRE-mediated mechanisms that modulate the action of receptor domains.

MATERIALS AND METHODS

Plasmids

RS-RTrα, RS-v-erbA, and tk-TREp-CAT were gifts from R. Evans (Salk Institute for Biological Studies, La Jolla, CA). The expression vectors contain rTRα cDNA or the gag-v-erbA oncogene under the transcriptional control of the RSV-LTR (14, 48). tk-TREp-CAT contains a synthetic palindromic TRE linked to a herpes simplex virus tk-CAT fusion gene (14). The T3-responsive CAT reporter constructs contain maTRE, llyTRE, rGH-TRE, or TREp linked to a MTV (ΔMTV) LTR-CAT fusion gene (ΔMTV-CAT) (54). The pPTP-CAT reporter construct was a gift from R. N. Eisenman (Fred Hutchinson Cancer Research Center, Seattle, WA) and contains a 450-base-pair Sau3A-BamHI fragment from clone 122, spanning all three TREs, cloned into pBLCAT3 (10). pRSV-Pit-1 was a gift from H. H. Samuels (New York University Medical Center, New York, NY) and contains a CDNA clone of the rat Pit-1/ GHI-1 gene linked to the RSV LTR (49). The RSH-TRβ construct contains the wild type hTRβ gene, and the RSSh-TRβ C122 > A expression plasmid contains an in vitro-generated mutant hTRβ gene (alanine instead of cysteine at position 122), both under control of the RSV LTR (64). The RSH-RRXα expression vector contains hRXα CDNA linked to the RSV LTR, ΔMTV-CAT contains a CAT reporter gene driven by the ΔMTV LTR promoter (48). PRSV-lacZ was obtained from M. Harkey (University of Washington, Seattle, WA) and contains the Escherichia coli β-galactosidase gene under control of the RSV LTR (60).

Microinjections and ELISAs

A lobe of ovary was surgically removed from an adult female Xenopus laevis and processed as described (51). Microinjections were performed according to published methods with modifications (51, 52). Stage V oocytes were microinjected with 20 nl plasmid DNA in TE (10 mM Tris-HCl, 1 mM EDTA), pH 8.0, into the nucleus by the "blind" injection method with the needle inserted in the center of the animal pole (27, 53). After microinjection, oocytes were incubated in OR-2 (51) medium for 20-24 h at 18 C in the presence or absence of 100 nM T3 (Sigma Chemical Co., St Louis, MO). Healthy oocytes (uniform pigment, not mottled) were homogenized singly in 200 μl Tris buffer, pH 7.5, and centrifuged at 10,000 × g in a microfuge for 4 C for 10 min. The supernatant was then centrifuged as before. This extract was used in the determination of CAT and β-galactosidase protein expression levels by ELISA according to the manufacturer's specifications (5 Prime-3 Prime, Inc., Boulder, CO). The concentration of total protein in extracts from single oocytes was adjusted to a concentration of 175-200 μg/ml by 1:1 dilution with dilution buffer. Microwells coated with rabbit polyclonal antibody specific to CAT protein or specific to the E. coli β-galactosidase protein (β-Gal) were incubated with extracts from microinjected oocytes, uninjected controls, and appropriate pure CAT or β-Gal protein standards. Biotinylated secondary antibody to CAT or β-Gal was then bound to the primary antibody-antigen complex. After washing, bound biotinylated antibody was quantitated colorimetrically by incubation with streptavidin-conjugated alkaline phosphatase and p-nitrophenyl phosphate as substrate. The wells were read at 405 nm against the reagent blank in a microtiter well reader (model EL311, Bio-Tek Instruments, Winocov, VT). For each assay, a standard curve utilizing four pure protein standards was prepared, to ensure that CAT or β-Gal concentrations in sample extracts fell within the linear range of the assay. All experiments were repeated two to three times with different animals since transcriptional activity may vary between different batches of oocytes.

ELISA for Total T3

Ten oocytes per sample, either incubated in 100 nM T3 for 20 h and subsequently washed three times in OR-2 or cultured in OR-2 only, were homogenized in 100 μl 0.25 M Tris buffer, pH 7.5, and centrifuged at 10,000 × g in a microfuge at 4 C for 10 min. The supernatant was then centrifuged as before. This extract was used in the quantitative determination of total T3, both bound and free hormones, in oocytes by ELISA (Enzymun-Test T3, Boehringer Mannheim, Auckland, New Zealand). Test tubes coated with streptavidin were incubated with oocyte extracts or T3 standards, together with anti-T3 antibody-herosinder peroxidase conjugate. T3 standards were prepared in 0.25 M Tris buffer, pH 7.5, at 0, 0.1, 1, 10, and 100 nM. Binding of sample T3 to anti-T3 antibodies was then competed by addition of biotinylated T3 polyhapten, which anchos previously unbound anti-T3 antibodies to the streptavidin-coated test tube wall. After washing, bound anti-T3 antibody-herosinder peroxidase conjugate was quantitated colorimetrically by incubation with 2,2'-azino-di-[3- ethyl-benzthiazolium sulfonate (6)]. The samples were
transferred to microcuvettes and read against buffer at 420 nm in a spectrophotometer. For each assay, a standard curve utilizing the five T₃ standards was prepared. Two independent determinations were carried out with oocytes from two different animals, and four replicates (10 oocytes per replicate) were assayed in each determination.

Preparation of Nuclear Protein Extracts from Ovine Anterior Pituitary Cells and Cytoplasmic Injection

Nuclear protein extracts were prepared essentially as described by Maxon et al. (39). All procedures were carried out at 4°C. Ten milliliters of sheep anterior pituitary cells maintained in primary cell culture (4.8 × 10⁷ viable cells/ml) were concentrated by centrifugation, washed in 0.1 M Tris, pH 7.5, and dialyzed for 15 min. The gray nuclei were collected from the interface between the two sucrose solutions. The nuclei were diluted 2-fold with a buffer containing 75 mM NaCl, 25 mM EDTA, 5 mM Tris, pH 7.5, 20 mM 2-mercaptoethanol, and 0.1 mM PMSF. Partially lysed nuclei and chromatin were collected by centrifugation at 10,000 rpm for 10 min. The chromatin-nuclei pellet was suspended in extraction buffer (0.45 M NaCl, 20 mM Tris, pH 7.0, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.1 mM PMSF) and vortexed for several minutes. The insoluble material was collected by centrifugation at 10,000 × g for 30 min. The supernatant was set aside, and the extraction and centrifugation were repeated on the residual nuclear material with extraction buffer containing 1 M NaCl and then 2 M NaCl. The extracts of all three salt washes were pooled and concentrated by precipitation with ammonium sulfate (36%).

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Appendix 2

Solutions

O-R2, pH 7.6 (working concentrations):

82.5 mM NaCl
2.5 mM KCl
1.0 mM CaCl₂
1.0 mM MgCl₂
1.0 mM Na₂HPO₄
5.0 mM Heps
3.8 mM NaOH

O-R2 working solution:

10 ml Stock A
10 ml Stock B
80 ml sterile dd H₂O

O-R2 Stock A (10X):

825 mM NaCl 4.820 g
25 mM KCl 0.186 g
10 mM Na₂HPO₄ 0.142 g
50 mM Heps 1.190 g
38 mM NaOH 3.8 ml (1M stock)*

* freshly made, 0.4 g/10 ml ddH₂O

O-R2 Stock B (10X):

10 mM MgCl₂ 0.203 g (or 10 ml of 100 mM)
10 mM CaCl₂ 0.147 g (or 10 ml of 100 mM)

NET-2:

50 mM Tris-HCl, pH 7.4
150 mM NaCl
0.05% NP-40 (add after autoclaving)