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Thyroid abnormalities and hepatocellular carcinoma in mice transgenic for v-*erbA*

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The v-*erbA* oncogene consists of an avian retroviral *gag* gene fused to a mutated thyroid hormone receptor. To define better its role as an oncogene in mammals and its ability to function as a dominant negative transcription factor, transgenic mice expressing v-*erbA* ubiquitously were generated. The effects of v-*erbA* are pleiotropic, tissue-specific and dose dependent. Mice have breeding disorders, abnormal behavior, reduced adipose tissue, hypothyroidism with inappropriate TSH response, and enlarged seminal vesicles. This provides an animal model consistent with the proposal that v-ErbA functions as a dominant negative receptor by transcriptional interference or squelching of normal receptors or associated proteins. Finally, male animals develop hepatocellular carcinoma, demonstrating that v-*erbA* can promote neoplasia in mammals.

Key words: adipose/liver/pituitary/receptor/retinoic

Introduction

The *gag*−v-*erbA* oncogene is the viral counterpart of the c-*erbA* gene which encodes chicken thyroid hormone receptor α (TRα) (Sap *et al.*, 1986; Weinberger *et al.*, 1986). TRα is a member of the family of nuclear hormone receptors which includes, among others, the thyroid (TR), vitamin D3 (VD3R), retinoic acid (RAR) and 9-*cis*-retinoic acid (retinoic X, RXR) receptors, all of which function as sequence-specific DNA binding, ligand-dependent transcription factors (Evans, 1988; Glass and Holloway, 1990). v-*erbA* consists of the retroviral *gag* gene fused to c-*erbA* from which it is distinguished by point mutations and deletions; as a consequence, v-ErbA does not bind hormone at physiologic concentrations (Sap *et al.*, 1986; Munoz *et al.*, 1988). In addition, v-ErbA still binds DNA in a sequence-specific manner, albeit with an altered affinity (Sap *et al.*, 1989; deVerniuel and Metzger, 1990; Bonde *et al.*, 1991). These mutations render v-ErbA capable of interfering with the normal transactivation function of ligand-bound TR on several different promoters (Munoz *et al.*, 1988; Damm *et al.*, 1989; Sap *et al.*, 1989; Desbois *et al.*, 1991; Chen and Privalsky, 1993; Hermann *et al.*, 1993; Köhne *et al.*, 1993; Yen *et al.*, 1994). Therefore, the v-ErbA protein is proposed to act as a constitutive repressor of specific gene transcription and has served as a prototype for dominant negative transcription factors.

v-*erbA* was found as one of two oncogenes encoded by the avian erythroblastosis virus (AEV-ES4), the second being v-*erbB* (Vennström and Bishop, 1982). AEV is an acutely transforming retrovirus, which causes rapid, fatal erythroleukemia when injected intravenously into susceptible chicks (Engelbreth-Holm and Rothe-Meyer, 1935). Following injection into the wing-web of young chicks, sarcomas develop locally whereas a lethal erythroblastosis develops later as virus spreads from the tumor. The expression of v-*erbB* is sufficient for the induction of both erythroleukemia and sarcomas, while v-*erbA* has previously not been shown to cause tumors (Engelbreth-Holm and Rothe-Meyer, 1935; Frykberg *et al.*, 1983). Instead, v-*erbA* potentiates the ability of v-*erbB* to transform chicken erythroblasts by inhibiting the spontaneous differentiation of normal and transformed erythroblasts, and decreasing the stringency of conditions required for growth in culture (Fuerstenberg *et al.*, 1992, and references therein). Furthermore, tumor formation *in vivo* by v-*erbB*-transformed fibroblasts is promoted by v-*erbA* (Gandrillon *et al.*, 1987).

Mutant thyroid hormone receptors found in individuals with the syndrome of generalized resistance to thyroid hormone (GRTH) also act as dominant negative transcription factors (Refetoff *et al.*, 1967). GRTH is usually a dominantly inherited syndrome of reduced tissue responsiveness to thyroid hormone and is due to point mutations in the human thyroid hormone receptor β (hTRβ) gene. v-ErbA has served as a paradigm transdominant negative thyroid hormone receptor for this syndrome.

To investigate the dominant negative characteristics of v-ErbA *in vivo* and to understand its effects on thyroid hormone-regulated cell growth and tissue development, we have created transgenic mice expressing the v-ErbA protein ectopically using the human β-actin promoter (hAP) (Gunning *et al.*, 1987; Prabir *et al.*, 1991). These transgenic mice have profoundly different abnormalities from those seen in individuals with GRTH. The mice exhibit hypothyroidism, reduced fertility, decreased body mass and behavioral abnormalities. Furthermore, male mice develop seminal vesicle abnormalities and hepatocellular carcinoma. This is the first demonstration that v-*erbA* is tumorigenic in a mammalian organism.

Results

Establishment of transgenic mice

To achieve ectopic expression, *gag*−v-*erbA* was cloned between the hAP and the SV40 polyadenylation site as shown in Figure 1. To verify that the appropriate Gag−v-ErbA protein was produced, Western blotting of whole

cell extract isolated from NIH-3T3 cells transfected with this construct was done with an anti-ErbA antibody that detected the appropriate 75 kDa protein (data not shown)

The injections into fertilized eggs and subsequent implantation into foster mothers resulted in 21 live pups, of which six male (lines A–F) and seven female (lines 1–6 and 8), carried the transgene. Several founders could not be studied (Table I). These founders either failed to reproduce (lines 1, 2, 5 and 6), died for reasons apparently unrelated to the transgene (lines 3 and 4), expressed very low levels of the transgene (line B) (Figure 2, left panel), or harbored the transgene at more than one integration site (line F) (Table I and data not shown). Therefore, founders A, C, D, E and 8 and their offspring were analyzed, and were found to transmit the transgene through the germline in a Mendelian distribution as revealed by Southern analyses (data not shown).

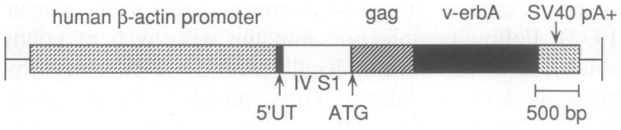

Fig. 1. DNA construct. The vector contains 3 kb of the hAP sequence, 78 nt of 5′ untranslated region (5′ UT) from the same gene, 832 nt of intervening sequence 1 (IVS1), 2.26 kb of the *gag*–v-*erbA* coding sequence and a 500 nt fragment containing the SV40 polyadenylation signal. The ATG indicates the start codon for the *gag*–v-*erbA* gene.

Expression of v-erbA in progeny animals

To verify that the transgene construct was expressed from the hAP promoter in a wide variety of tissues, Northern blotting and *in situ* hybridization experiments were done. Furthermore, the levels of transgene expression in different lines were compared as variable severity of some phenotypes was evident (see below). Northern analysis of RNA extracted from whole brain using a *gag*-specific riboprobe (Figure 2, left panel) showed that line A expressed the highest level of v-ErbA, line B the lowest, and that in normal mice no hybridization could be demonstrated. Analysis of tissues from line A animals (Figure 2, right panel, and data not shown) showed v-ErbA expression in all tissues tested, including adipose, cerebrum, cerebellum, kidney, liver, ovaries, testes, seminal vesicles, thyroid, pituitary and muscle. In addition, males and females within the same line showed the same level of transgene expression (Figure 2, right panel)

The above data were confirmed and extended by *in situ* hybridization. For this, newborn pups from lines A, C, E and 8 as well as adult brain and pituitary gland from line A, were sectioned and hybridized with a *gag*-specific probe. Transgenic mice had a strong hybridization signal

Table I. Founder characteristics

Founder animal	Relative expression	F1	F2	Homozygotes	Characteristics and reproductive ability
Males					
A	high	+	+	rare	female homozygotes sterile; male premature sterility
B	very low	+	+	+	neonatal lethal as homozygote
C	low	+	+	+	variable infertility in female homozygotes; male premature sterility
D	high	+	−	−	two F1 males both sterile
E	high	+	+	rare	female homozygotes sterile; male premature sterility
F	low	+	−	−	two integration sites
Females					
1	n.d.	−	−	−	sterile
2	n.d.	−	−	−	sterile
3	n.d.	−	−	−	premature death
4	n.d.	−	−	−	death secondary to pregnancy
5	n.d.	−	−	−	no viable offspring
6	n.d.	−	−	−	no viable offspring
8	moderate	+	rare	−	founder produced one transgenic male; F1 male fathered 12 pups (continuous mating); F2 female produced no viable offspring

+, progeny born; −, no progeny; n.d., not done

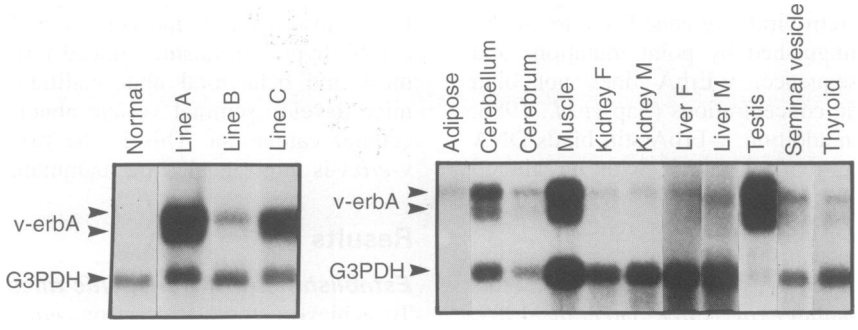

Fig. 2. Expression of v-*erbA* RNA. Northern blots of polyadenylated RNA from adult brain of lines A, B, C and normal animals (left) and various tissues from line A female and male animals (right) are shown. M, male; F, female; G3PDH indicates the signal for glyceraldehyde 3-phosphate dehydrogenase as the control for RNA loading; v-*erbA* indicates the expected 2.6 kb band of *gag*–v-*erbA* RNA.

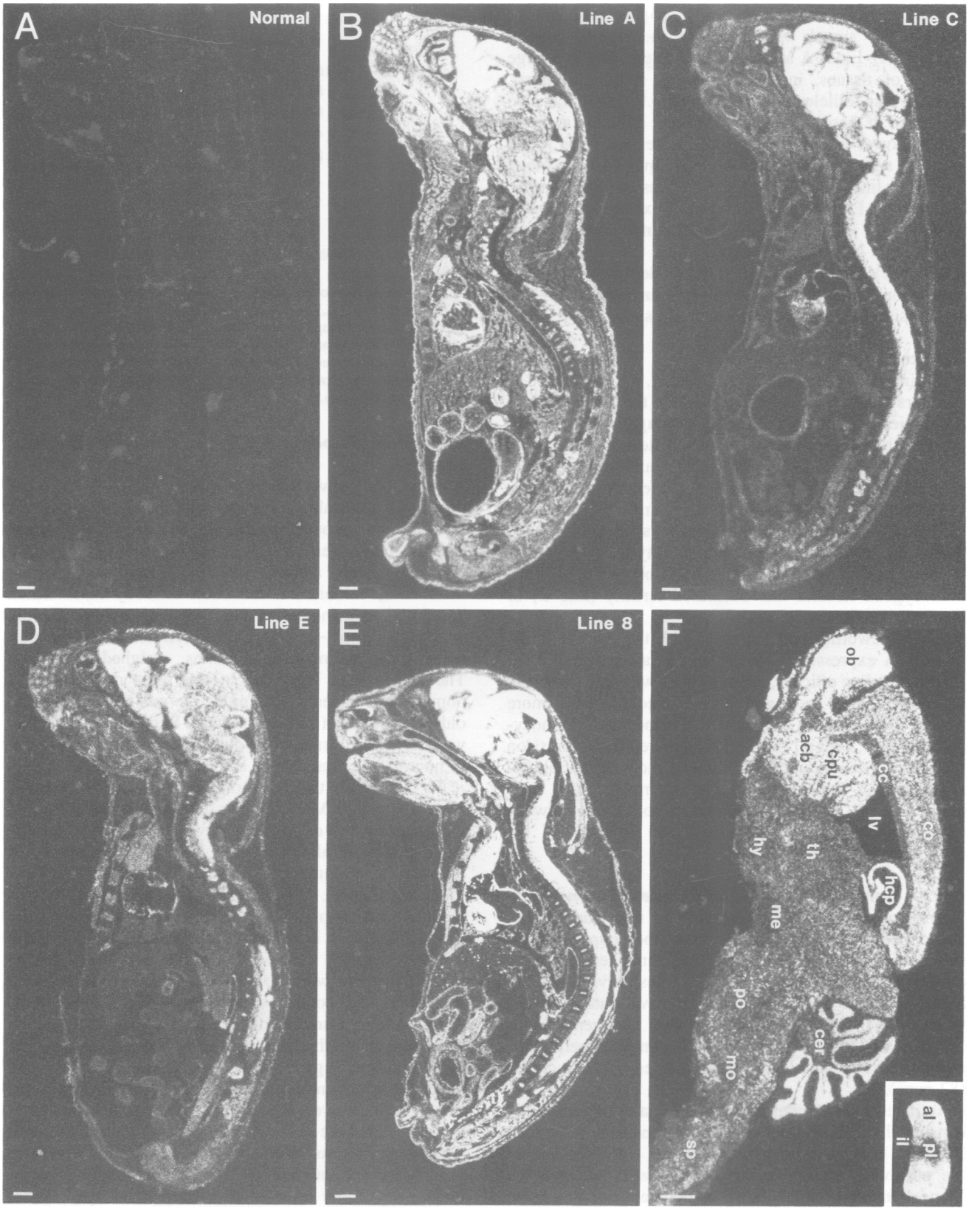

Fig. 3. Transgene expression in newborn pups and adult brain. Shown are film autoradiograms of *in situ* hybridization from sections of newborn normal (**A**) and transgenic mice (line A, C, E and 8) (**B**–**E**), and of the adult brain and pituitary from line A (**F** and **inset**) after hybridization with an oligonucleotide probe to the *gag* portion of v-*erbA*. acb, nucleus accumbens; al, anterior lobe; cc, corpus callosum; cer, cerebellum; co, cerebral cortex; cpu, caudate putamen; hcp, hippocampus; hy, hypothalamus; il, intermediate lobe; lv, lateral ventricle; me, mesencephalon; mo, medulla oblongata; ob, olfactory bulb; pl, posterior lobe; po, pons; sp, spinal cord; th, thalamus. Bar = 1 mm.

in the central nervous system (CNS) (Figure 3B–F), whereas no signal was found in the normal animal (Figure 3A). Lines A and 8 had high expression in a large variety of tissues, including muscle, lung, gut, heart, thymus, hair follicles and bone marrow. v-ErbA was also expressed in these tissues in other lines, although at lower levels. Lower levels of transgene expression were found in brown adipose tissue and liver in all lines. In comparison with other lines, line A had higher expression in all tissues (Figure 3B–E); however, the overall expression patterns were similar in the different lines, and confirmed the results from Northern analyses.

Ribonuclease protection assays demonstrated that expression began at least as early as embryonic day 9.5 and persisted throughout life (data not shown).

Reduced ability to reproduce

Offspring and founders from lines A, C, D, E and 8 founders appeared healthy; however, the majority had difficulties in producing offspring although caged continuously with fertile animals of the opposite sex (Table I and data not shown). Male animals from all lines generally showed a limited ability to impregnate female at 6 months of age and were sterile by 9 months. Female offspring had similar abnormalities. The heterozygous females showed decreased fertility and premature sterility (lines A and E) or were unable to produce viable offspring (line 8). All homozygous females had decreased fertility (line C) or were sterile (lines A, C and E).

It is noteworthy that the lines with the highest levels of v-ErbA expression (lines A and 8) also exhibited the lowest breeding efficacy, whereas lines with low transgene expression, such as B and C, produced offspring more readily (Table I, data not shown). Furthermore, homozygous animals, with a 1.3- to 1.5-fold increase in expression of v-ErbA (data not shown) showed more severe abnormalities than their heterozygous littermates and were usually sterile. Therefore, there was a correlation between expression of the transgene and the severity of the phenotype.

v-erbA transgenic females are inadequate mothers

Transgenic females that were able to reproduce, either abandoned or consumed all or some of their pups. Figure 4 shows that when heterozygous transgenic females were crossed to normal males, 26 of 73 pups survived giving a 36% average (12.5% median) survival rate, whereas when normal females were mated with transgenic males 227 pups of 230 survived (98.7%). In control experiments, normal females mated with normal males, 100% survived (82 pups).

To determine whether transgenic females preferentially consumed or abandoned normal or transgenic offspring, Southern blot analysis was performed on tail DNA of the surviving pups. The results showed the expected Mendelian ratio: half of the survivors were transgenic and half were normal. This suggests that v-*erbA* affects the behavior of transgenic females and causes them to abandon or consume their pups, irrespective of whether their pups are normal or transgenic.

Again, females from the higher expressing lines A and 8 were more affected than those of the lower expressing lines C and E.

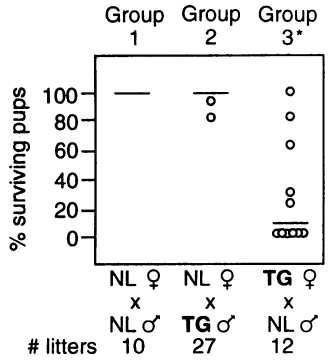

Fig. 4. Transgenic mothers abandon or consume their pups. Transgenic (TG) and normal (NL) mice were mated as indicated. The median value for the percentage of pups that survived for >3 days was calculated. The median is indicated by a bar and values outside of the median are represented as circles. * $P \leq 0.001$ when comparing group 3 with group 1 or group 2 (based on Kruskal–Wallis multiple comparison *Z*-values).

Reduction of body mass due to decreased adipose tissue

A correlation between decreased body mass and transgene expression was also seen. All transgenic male animals and all homozygous animals, except those of line C, had reduced body weight. The data (Figure 5A) show that transgenic male animals, in comparison with normal littermates, exhibited a reduction of body mass which correlated with increasing age: younger male transgenic animals weighed ~15% less than their normal littermates, while older animals, 9.5 months, weighed 30% less. The reduction in body weight was more pronounced in homozygous males: a 50% reduction was seen in 3 month old line A males (data not shown). Reduced body weight was not evident in heterozygous females but was present in homozygous females as shown in Figure 5B.

To investigate the cause of weight reduction, animals were autopsied. Male animals had a near total lack of white adipose and diminished brown adipose tissue without any other gross abnormalities, whereas the females had normally distributed but markedly decreased body fat (data not shown). Weighing of dissected tissue showed that the content of brown fat was generally reduced by 75–80% in line A heterozygous males in comparison with controls, and by 75–85% in white fat. It should be noted that there were no significant differences between transgenic and control skeletal or muscle dimensions. Additionally, young animals undergo a normal growth spurt suggesting that feeding behavior is not altered. The data therefore suggest that v-*erbA* reduces brown and white fat adipogenesis in a dose dependent fashion since lines with high levels of v-ErbA expression lacked adipose tissue whereas the low expressing line C was unaffected. Also, homozygous males of lines A and E were more profoundly affected than heterozygous littermates. Finally, males were more affected than females as only homozygous females lacked adipose tissue.

Hypothyroidism and inappropriate TSH response

To explore the possibility that the decreased fertility, poor mothering and reduced adipose tissue were due to abnormal thyroid or pituitary hormone levels, a number

of hormone determinations were done. T4 and T3 were assayed in transgenic animals of lines A and C and compared with the levels found in euthyroid littermates and methimazole (1-methyl-2-mercaptoimidazole, MMI) induced hypothyroid animals. We chose to perform hormone assays on lines A and C as line A had 2-fold higher expression of v-ErbA, decreased body mass and severe reproductive abnormalities including sterility in homozygous females, whereas line C had normal body weight and less severe reproductive abnormalities. If line A had hormone abnormalities not present in line C, this would suggest that a hormone disturbance was responsible for the severity of the phenotype in line A animals. Conversely, if the two lines had similar hormone profiles it would suggest that these phenotypes are caused by an influence of v-ErbA on specific tissues rather than by inducing hormonal abnormalities.

The animals tested were 25–34 days old for measurements of total T4 and total T3 whereas free T3 and free T4 was measured in males aged from 34 days to 6 months and in females aged from 34 days to 20 months. Figure 6A and B shows that the transgenic animals exhibited a large variation in T3 and T4 levels. However, the median level was significantly lower than that of controls. In addition, severely affected transgenic animals had levels as low as those in the chemically induced hypothyroid animals. The frequency of severe hypothyroidism was similar in the line A and C animals and showed no sex predilection.

The animals described above had been kept on a diet rich in iodine, and we therefore tested if the hypothyroidism could be enhanced by a low iodine diet. Selected normal and transgenic littermates and the MMI treated animals were placed on a low iodine diet and their serum assayed. Figure 6A and B (compare crossed and filled symbols) shows that most transgenic and MMI treated animals, but not the normal control animals, had further decreases in their thyroid hormone levels, most notably T4.

Hormone levels in lines D, E and 8 were not analyzed with matched controls due to inadequate numbers of pups caused by the breeding problems. However, results from limited studies were consistent with those described for lines A and C.

The data thus show that both males and females of lines A and C exhibited a similar degree of hypothyroidism and that the severity of hypothyroidism was not correlated with the aforementioned phenotypes. Only line A animals had reduced adipose tissue and severe breeding abnormalities, confirming that hypothyroidism alone was not responsible for the more severe phenotype seen in line A animals.

Hypothyroidism normally results in a rise in anterior pituitary gland TSH expression which then causes increased production of thyroid hormones by the thyroid gland. Therefore, radioimmunoassays were done to determine TSH levels in transgenic and normal littermates. For this, strictly matched hypothyroid transgenic (of lines A and C) and normal animals were kept on a low iodine diet to enhance hypothyroidism. The levels were also compared with those found in animals made hypothyroid by treatment with MMI. Figure 7 shows that the range of TSH levels in 14 transgenic animals paralleled those found in seven controls, whereas the MMI treated animals had significantly elevated levels. Further analysis of >50

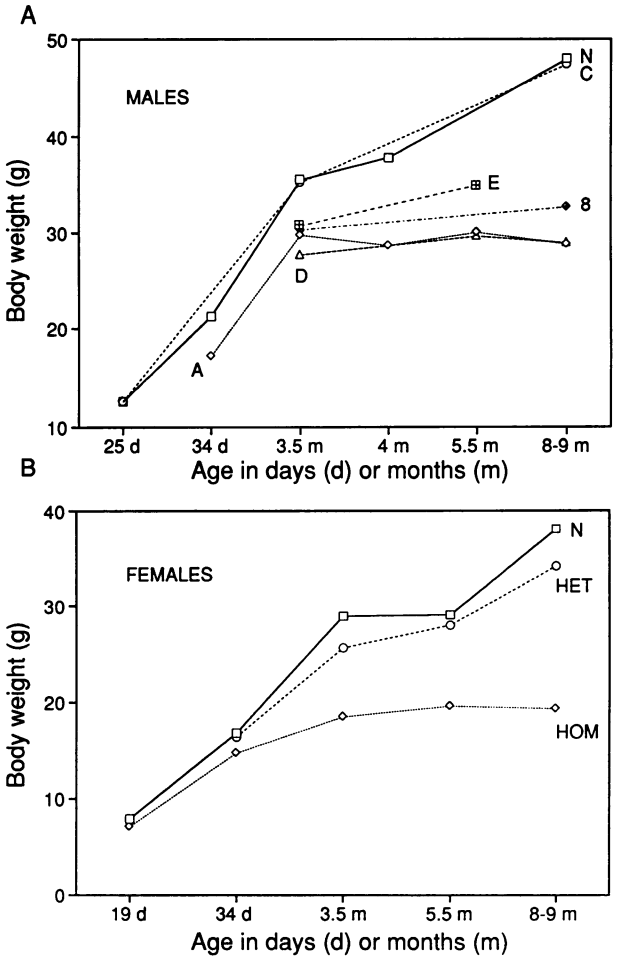

Fig. 5. Decreased adipose tissue in transgenic mice. Control and transgenic male (**A**) and female (**B**) animals from the various lines were weighed at regular intervals. N represents normal littermates, HET represents heterozygote females and HOM represents homozygote females. Each point represents the average weight of between two and eight transgenic animals or between two and 10 normal animals.

animals including those with severely reduced T4 and T3 levels gave similar results (data not shown).

The levels of several other anterior pituitary hormones were also determined. The serum levels of growth hormone (GH), luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured in over 50 animals and no differences from the levels found in control animals were found (data not shown).

To evaluate the cause of thyroid dysfunction further, sections of pituitaries from hypothyroid animals were either (i) stained with histologic dyes and inspected for morphological abnormalities or (ii) used for *in situ* hybridization using an oligonucleotide probe specific for the *gag* portion of v-*erbA*. No obvious changes in morphology were seen (data not shown); however, the transgene was expressed in the anterior pituitary gland, including regions known to produce TSH (Figure 3F, inset, and data not shown).

We then performed histologic analyses of thyroid gland morphology in line A, C and D animals. The results showed (Figure 8A–E) that the follicular structures producing thyroid hormone precursors were disorganized, of uneven sizes, and had little interstitial tissue when compared with

normal control glands. Additionally, some follicles had flattened epithelium and were distended by colloid (Figure 8D) whereas others had colloid matrixes which were lighter in color and often filled with debris (Figure 8E).

Our results therefore indicate that v-ErbA induces hypothyroidism by at least two mechanisms. First, it interferes with the ability of the thyroid gland to produce normal amounts of thyroid hormones in the presence of normal levels of serum TSH. Second, it blocks the increase of pituitary TSH in response to hypothyroidism.

Enlarged seminal vesicles and hepatocellular carcinomas

To determine if v-ErbA dysregulated cell growth or had neoplastic properties, male and female animals of lines A, C and E were sacrificed and autopsied. The results showed that seven of 17 male animals examined had grossly enlarged seminal vesicles (Table II). It was evident that the abnormality was progressive: older males had more severe enlargements than younger ones (Figure 8F–H). Microscopic inspection of the enlarged seminal vesicles revealed that the normal convoluted architecture was lost, apparently as a result of severe distension by excessive glandular secretions. However, the glandular epithelia appeared normal, but as a result of the excessive seminal vesicle fluid, in some areas a single epithelial layer abutted the muscular wall (Figure 8H). The glandular secretions stained similarly to those of control animals but the staining was was 2- to 10-fold more abundant (Figure 8H). There was no evidence that the excess material accumulated from an outflow obstruction.

In addition to seminal vesicle abnormalities, the male v-*erbA* transgenic mice developed hepatocellular carcinomas. Eleven of 19 transgenic males but only one of 12 normal males had liver tumors (Table II). In contrast, one of 20 transgenic females and none of 12 normal females developed tumors, suggesting that tumor development was specific to transgenic males. Histological sections of several liver tumors from different lines revealed moderately well-differentiated hepatocellular carcinoma as shown in Figure 8I and J. These tumors rarely metastasized except in animals which had appeared ill for more than 6 months prior to sacrifice. Northern blotting of tumor tissue as compared with normal liver showed no significant difference in the level of v-ErbA expression (data not shown). Therefore, in male animals the v-*erbA* transgene predisposed normal hepatocytes to undergo malignant transformation. This transformation generally occurred after 9 months of age and the likelihood of transformation increased with increasing age.

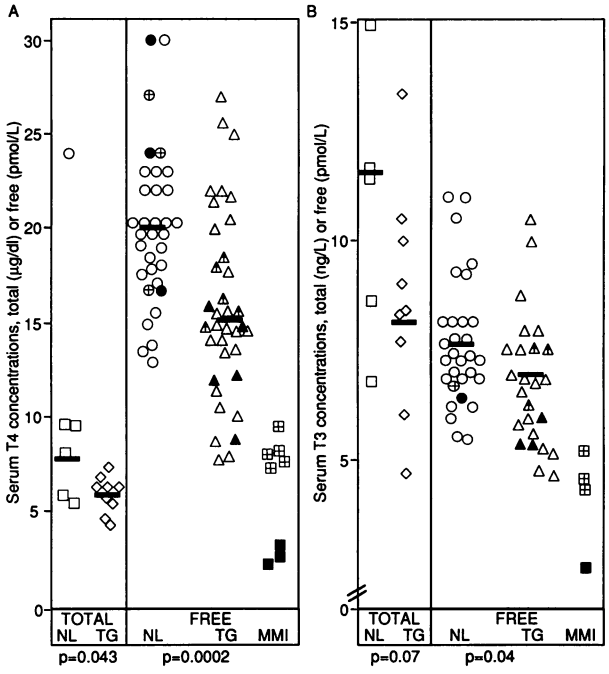

Fig. 6. Hypothyroidism in v-*erbA* transgenic animals. T4 and T3 levels were determined for transgenic animals (TG) and at least one normal littermate (NL) matched for sex, age, mating and number of pregnancies. (**A**) Control total T4 (□), transgenic total T4 (◇), control free T4 (○), transgenic free T4 (△) and MMI control total T4 (□) are as indicated. Crossed and filled symbols represent values obtained from animals before and after a low iodine diet respectively. Mean values are indicated by bars and *P* values are as indicated for comparisons of both total and free hormone values between control and transgenic animals based on Student's paired *t*-tests. (**B**) Control total T3 (□), transgenic total T3 (◇), control free T3 (○), transgenic free T3 (△) and MMI control total T3 (□) are as indicated. Crossed and filled symbols represent values obtained from animals before and after a low iodine diet respectively. Mean values are indicated by bars and *P* values are as indicated for comparisons of both total and free hormone values between control and transgenic animals using Students paired *t*-tests.

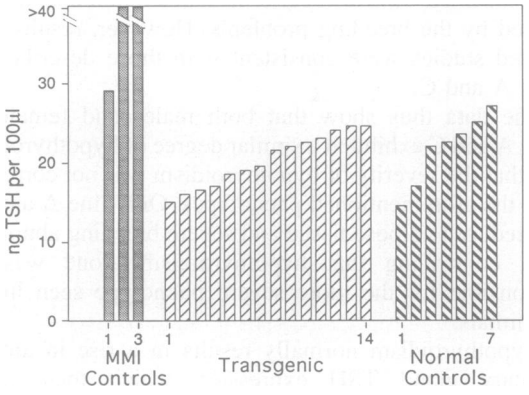

Fig. 7. Lack of the appropriate TSH elevation in response to hypothyroidism. Serum TSH values of three MMI-treated, 14 transgenic and seven control animals are compared. Each bar represents one animal. Transgenic and control animals represent littermates and corresponding T4 and in some cases T3 analyses are shown in Figure 6.

Discussion

v-ErbA acts as a dominant negative transcription factor

The function of v-ErbA as a dominant negative transcription factor has been demonstrated in several experimental model systems. It is suggested that v-ErbA exerts a dominant negative effect by heterodimerization with RXR, thereby competing for DNA binding sites or by squelching factors required for normal TR and RAR mediated gene regulation (Zenke *et al.*, 1988; Damm *et al.*, 1989; Sap *et al.*, 1989; Barettino *et al.*, 1993; Chen and Privalsky, 1993; Saatcioglu *et al.*, 1993; Zavacki *et al.*, 1993). These data also suggest that v-ErbA repression is mediated via

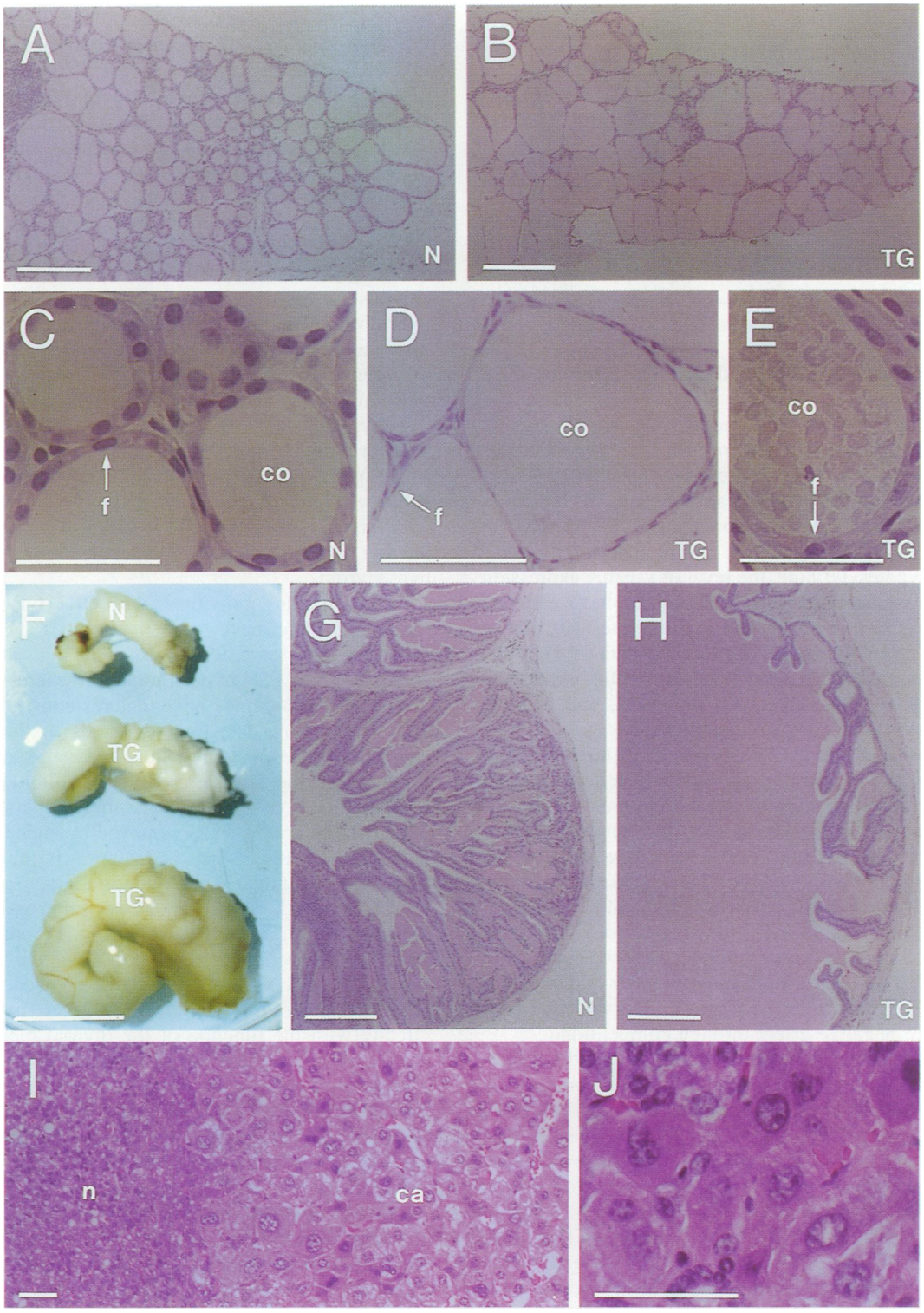

Fig. 8. Tissue pathology of v-*erbA*-transgenic animals. (**A**–**E**) Hematoxylin and eosin stained sections of thyroid glands from normal (N) and transgenic (TG) mice. Overall morphology is seen in panels A and B. (C–E) Comparison of thyroid follicles of normal and transgenic mice. Note in D, the distended colloid space (co) and flattened follicular epithelium (f) in comparison with the normal thyroid epithelial morphology (C). Some areas of the transgenic thyroid gland contain follicles filled with debris (E). (**F**) Comparison of two grossly abnormal seminal vesicles (lower two marked TG) with a normal one (upper marked N). The normal seminal vesicle (N) is from a 12 month old normal male and the middle from a line A littermate. The lower seminal vesicle is from a 18 month old line C male. (**G** and **H**) Hematoxylin and eosin stained sections of normal seminal vesicle (N) and transgenic seminal vesicle (TG) respectively. Note in panel H the markedly increased secretory material compressing the glandular epithelium. (**I** and **J**) Hematoxylin and eosin stained sections of the hepatocellular carcinoma (ca). In panel I the hepatocellular carcinoma occupies the majority of the figure and the carcinoma cells have marked variation in shape and are disposed in a sinusoidal network. Minimal residual normal hepatic parenchyma is visible to the left (n). Note the variation in nuclear shape and coarse chromatin illustrated in panel J. Bar in A, B, G and H = 500 μm; bar in C–E, I and J = 100 μm; bar in F = 1 cm.

Table II. Transgenic male animals develop seminal vesicle dysfunction and hepatocellular carcinoma

Animals	Incidence of abnormal s.v.	Age at autopsy (months)	Average age (months) s.v./liver	Incidence of liver tumors
Transgenic males	7/17 (41%)	9.5–23	16.4/16.1	11/19 (58%)
Control males	0/12 (0%)	12–21.5	16.3/16.3	1/12 (8%)
Transgenic females		13.5–22.5	n.a./16.0	1/20 (5%)
Control females		12–22	n.a./17.2	0/12 (0%)

s.v., seminal vesicle. n.a., not applicable.

thyroid response elements, and that the action is dose dependent.

In accordance with this model, we found a good correlation between the severity of the abnormalities and the level of v-ErbA expression. This was seen in the differences in the breeding abilities and the reduction of adipose tissue. Furthermore, clear differences in penetrance of the disorders were observed between hetero- and homozygotes. Of note, the level of v-ErbA expression relative to endogenous TRα1 was difficult to estimate accurately as the transgene mRNA co-migrates with mouse TRα2 mRNA in Northern analyses. The data (not shown) nevertheless indicate that the level of v-ErbA expression is substantially lower than that of TRα2, and may be lower than the TRα1 levels in some tissues.

The cause of these disorders appears different in each case. The breeding disorders are likely to be multifactorial but did not correlate with hormone abnormalities or with an inability to lactate. In males, the seminal vesicle abnormality could account for the premature sterility. The decreased adipose tissue was not due to inadequate growth hormone levels and, as mentioned, no significant differences in skeletal or muscle dimensions were observed. Therefore, v-ErbA influenced adipogenesis in both brown and white fat in a dose dependent manner and provides evidence consistent with the proposal that v-ErbA exerts its dominant negative effect through competition.

The dose dependence for elicitation of the aforementioned disorders contrasts with the situation in liver, seminal vesicle and thyroid hormone level abnormalities. The latter were seen in all founder lines, suggesting that even very low levels of v-ErbA expression are sufficient to cause a phenotype, whereas adipose tissue, for example, had a higher threshold.

It is interesting to note that in mice lacking a functional RARγ (Lohnes *et al.*, 1993) the seminal vesicle glandular epithelium undergoes squamous metaplasia whereas other vitamin A sensitive epithelia are unaffected. It is therefore possible that seminal vesicle epithelium is sensitive to v-ErbA because v-ErbA can more readily interfere with the action of limiting transcription factors, perhaps in the retinoid pathway.

Effects of v-erbA in transgenic mice and of mutant TRβ in GRTH

The ability of v-ErbA to affect specific cells is of particular interest with regard to the hypothyroidism in the transgenic animals. Three tissues are involved in the regulation of thyroid hormone production. The production in the pituitary of TSH, the major regulator of T3/T4 production in the thyroid gland, is normally repressed by liganded thyroid hormone receptors whereas TSH release from the pituitary is induced by thyrotropin releasing hormone (TRH), produced in the hypothalamus. Also TRH production is negatively regulated by thyroid hormones.

Our results suggest that the hypothalamic–pituitary–thyroid axis of hormone regulation is perturbed at more than one level in the hypothyroid transgenic animals: TRH and TSH levels were normal and not elevated as would have been expected in hypothyroid animals, as evidenced by immunochemistry and *in situ* hybridization (Figure 3F, inset, and data not shown). This indicates that v-ErbA interferes, directly or indirectly, with the regulation of TRH and/or TSH gene expression. In this context it is noteworthy that the mutated TRβ present in individuals with GRTH is unable to suppress efficiently anterior pituitary hormone production of TSH in response to the patient's hyperthyroidism. This indicates that the defective TRs (mutant TRβ and v-ErbA) can interfere with the regulation of the TRH and/or TSH genes, despite the fact that the patients are hyperthyroid and the animals hypothyroid.

Although many individuals with GRTH and the v-*erbA* transgenic mice have normal serum TSH levels, the thyroid glands from GRTH individuals show abnormalities consistent with TSH hyperstimulation whereas those from v-*erbA* transgenic mice have features consistent with low TSH stimulation. The thyroid glands of the mice have enlarged follicles, flat epithelium and well stained colloid. Furthermore, the debris present in the colloid is consistent with fragments of apical plasma membrane shed into the lumen which are normally removed by endocytosis along with thyroglobulin; when endocytosis is inhibited, these fragments accumulate. Such abnormalities are present in rodents when thyroid hormone secretion is inhibited as seen after experimentally decreased TSH production, when TSH sensitivity is decreased, or when the intracellular effect of TSH is interfered with (Nilson, 1991, and references therein). Therefore, v-ErbA may interfere directly with the function of the thyroid gland follicular epithelium and suggests that TRs, expressed in the thyroid gland (Akiguchi *et al.*, 1992), may have functions which remain to be elucidated. Alternatively, the bioactivity of TSH may be abnormal, as has been suggested to be the case for GRTH (Persani *et al.*, 1994).

GRTH patients often have subtle behavioral abnormalities that manifest as hyperactivity, learning disabilities and attention deficit–hyperactivity disorder (Hauser *et al.*, 1993). Although we found no specific abnormalities on gross tests of mental function, the inability of transgenic mothers to perform the complex task of caring for their pups may represent a behavioral abnormality.

In conclusion, the phenotype of the transgenic mice is consistent with a transdominant negative effect of v-ErbA. However, the phenotype differs in many but not all aspects from the symptoms of the GRTH patients, which in part may be due to differences in tissue expression of the respective genes.

v-erbA as an oncogene

v-*erbA*, in a retroviral context, is known to augment the transforming potential of v-*erbB*, v-*src*, v-*fps*, v-*sea*

and v-Ha-*ras* in avian erythroblasts by shifting their sarcomagenic spectrum into a more leukemogenic one, and was the first example of an auxiliary oncogene which could not directly transform cells (Kahn *et al.*, 1986). Recent studies have also indicated that v-*erbA* may contribute to neoplasia by interfering with the AP-1 pathway. For instance, v-*erbA* abrogates the inhibitory activity of TRs and RARs on AP-1-induced transactivation of the interstitial collagenase gene promoter (Desbois *et al.*, 1991; Zhang *et al.*, 1991; Konih *et al.*, 1992; Pfahl, 1993).

Our results show that v-*erbA* in mice can promote tumorigenesis, induce enlarged seminal vesicles and inhibit the accumulation of adipose tissue. However, the male-specific effects of v-*erbA* were unexpected. This suggests that estrogen may block v-*erbA* action or that androgens may potentiate it. Complex interactions between ligand bound estrogen receptor (ER) and c-*fos* transcription showing reciprocal antagonism between estrogen–ER complexes and c-Fos have been described (Ambrosino *et al.*, 1993). This raises the possibility that v-*erbA* abrogation of hormone dependent inhibition of AP-1 transcription is interrupted by liganded ERs, thus protecting female mice from its oncogenic effect. However, several lines of evidence suggest that testosterone acts as a permissive factor in the development of hepatocellular carcinoma. A correlation between hepatocellular carcinoma, induced by a variety of known carcinogens, and high endogenous or exogenous circulating levels of testosterone has been shown (Johnson, 1976; Yu and Chen, 1993). Therefore, it is possible that v-ErbA cooperates with androgens to render male animals prone to developing hepatocellular carcinoma. Finally, a male-specific occurrence of hepatic carcinoma in transgenic mice is not without precedent: only male transgenic mice overexpressing transforming growth factor α (TGFα) develop tumors (Jhappan *et al.*, 1990; Sandgren *et al.*, 1990).

The original identification and cloning of AEV and subsequent studies of v-*erbA* in conjunction with multiple transforming oncogenes established v-*erbA* as the first example of an auxiliary oncogene which could not directly transform cells. Our results suggest that although the precise mechanism by which v-*erbA* induces tumors is unclear, it is evident that v-*erbA* can act as a tumor inducing agent in mammals.

Materials and methods

Plasmids

The hAP/v-*erbA* transgene was constructed as follows. A 2.26 kb *Eco*RI fragment consisting of the coding region of the viral *gag* gene fused to v-*erbA* was isolated from pKCR-2 (Sap *et al.*, 1989), purified and filled in using the Klenow fragment of *E.coli* DNA polymerase I according to standard protocols (Sambrook *et al.*, 1989). This fragment was cloned between the blunted *Sal*I and *Hin*dIII sites of the human β-actin construct described previously (Gunning *et al.*, 1987). The final construct consists of 3 kb of 5′ flanking sequence from the human actin gene, exon 1 containing 5′ untranslated sequence, the first intron with its 3′ splice site followed by the v-*erbA* sequence, and terminated with an SV40 polyadenylation site. The vector has been shown previously to give adequate expression in transgenic mice (Prabir *et al.*, 1991).

To remove vector sequences prior to injection, cesium chloride purified hAP/v-*erbA* plasmid was digested with *Eco*RI and the 6.67 kb hAP/v-*erbA*/polyadenylation site *Eco*RI fragment was purified by agarose gel electrophoresis, dissolved in injection buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4), and finally filtered through a 0.2 μm filter (Dynagard) to remove particles before injection.

Generation and analysis of transgenic founders and offspring

To generate transgenic mice the purified *Eco*RI fragment was injected at 2 μg/ml into the male pronucleus of fertilized C57BL/6J × CBA/J F1 eggs according to the procedure of Hogan *et al.* (1986). Microinjected eggs were then transferred into the oviducts of pseudopregnant foster mothers. Offspring were analyzed by cleaving tail DNA with *Bam*HI and *Sac*I to release the transgene, followed by Southern analyses. Positive animals were mated, and tail DNA from their offspring was analyzed as above. To ascertain that transgenic founders transmitted single integration events, their F1 offspring DNA was additionally analyzed after cleavage with *Eco*RV and *Xba*I. The blots were hybridized with either a chicken TRα (Sap *et al.*, 1986) or an avian *gag*-specific probe; the latter corresponds to bases 1769–2215 of pCR-1 (Jansson *et al.*, 1987).

Southern blotting or slot blotting was done to determine copy number, heterozygosity and homozygosity. Probed filters were subjected to PhosphorImager analysis and quantification of signal strength. For blots probed with a cTRα probe, a ratio comparing transgene signal with cross-reacting endogenous mouse TR probe was used. For slot blot analysis, the *gag*-specific probe was used followed by stripping of the membrane and reprobing with a PCR generated, ^{32}P-labelled *G3PDH* probe (Clonetech), and the ratio of *gag* signal to *G3PDH* determined.

Analysis of transgene expression

To verify that the Gag–v-ErbA protein was expressed, extracts from cells transfected with the construct were subjected to Western blotting using a c-ErbA-specific antibody which detects the 75 kDa Gag/v-ErbA protein.

RNA. To evaluate temporal and tissue-specific expression of the transgene, ribonuclease protection assay (RPA), Northern blotting and *in situ* hybridization were performed. Total RNA was obtained according to the method of Chomczynski and Sachi (1987). Polyadenylated RNA was obtained as described by Vennström and Bishop (1982). RPA was performed using a c-*erb*A-specific probe previously described (Forrest *et al.*, 1990) to detect a 333 bp fragment. Twenty μg of total or 1–10 μg of poly were used for Northern blotting and 6 μg of total or 1–2 μg of poly(A)$^+$ RNA were used in RPA. The Northern filters were probed with either a full-length mouse TRα-1 cDNA probe or a *gag*-specific riboprobe corresponding to base pairs 1762–2161 of pCR-1. Hybridizations with riboprobe were done as described previously (Sideras *et al.*, 1992) with the labelled probe at ~3 × 10^8 c.p.m./10 ml of buffer. After overnight hybridization at 65°C the filters were washed three times in 0.1% SDS, 0.1 × SSC for 30 min followed by one wash in 1 × SSC for 15 min. The PCR-generated *G3PDH* probe was used as a control probe for RNA loading and hybridization with this probe and the mTRα-1 cDNA probe were done at 42°C overnight.

In situ hybridization. Oligonucleotide probes were designed using MacVector software (IBI, New Haven, CT), and regions were selected based on the optimum ratio of G+C/total nucleotide numbers (50–65%) and minimal homology (not greater than 75%) with eukaryotic sequences in the GenBank database. Oligonucleotide probes were synthesized (Scandinavian Gene Synthesis AB, Köping, Sweden) complementary to nucleotides 5′-AAGGGGGAGGAAATGGGAGAGACAACTGTGC-AGCGAGATGCGAAGATG-3′ of pCR-1 and 3′ end-labelled using terminal deoxynucleotidyltransferase (Amersham Ltd, Amersham, UK) and [^{35}S]dATP (New England Nuclear, Boston, MA) and subsequently purified on Nensorb-20 (NEN) columns.

Normal and transgenic newborn mice, adult brain and pituitary were frozen on dry ice and sectioned at 14 μm in a cryostat. *In situ* hybridization experiments were performed as previously described (Young, 1990; Dagerlind *et al.*, 1992). Briefly, sections were air-dried and incubated with 10^6 c.p.m. of probe in humidified boxes at 42°C for 18 h. Sections were washed, dehydrated and finally apposed to Hyperfilm-β_{max} autoradiography film (Amersham Ltd, Amersham, UK) for 1–3 weeks. For control purposes, adjacent sections were hybridized with radiolabelled probe in the absence or presence of an excess (100 times) of unlabelled probe.

Hormone assays

Whole blood was obtained by (i) decapitation and collection of blood from the carotid arteries, (ii) tail bleeding or (iii) by anesthesizing mice intraperitoneally (i.p.) with sodium pentobarbital (85 mg/kg body wt) and collecting blood from the inferior vena cava and by cardiac puncture. Blood was allowed to clot and was centrifuged and the serum was removed and stored at −20°C until assayed.

For some assays the animals were maintained on a low iodine diet

for 6 weeks to 6 months. Assays were done on blood obtained immediately before the switch to this diet, and after 1, 2 and 6 months. The low iodine diet consisted of Foder R584 obtained from AB Analycen, Lidköping, Sweden.

Total T4 and T3 were measured according to the protocol described previously (Iniguez *et al.*, 1992). Free T3 and T4 were measured using a commercially available direct competitive radioimmunoassay from Amersham (Amerlex-MAB* FT4 and FT3 Kit).

TSH assays were done with Amersham's rat TSH (rTSH) ^{125}I assay kit according to the protocol except that the TSH standard curve values were based on mouse reference preparation #AFP-517-1.8MP obtained from Dr A.F.Parlow (UCLA).

GH, LH and FSH assays were performed using Amersham's rat GH (rGH), LH (rLH) and FSH (rFSH) ^{125}I assay kits according to the protocol.

Tissue analysis and autopsy

Animals were sacrificed either by decapitation or by lethal injection of sodium pentobarbital either at specific time points or when they appeared ill. Tissues were dissected and weighed and/or frozen on dry ice and stored at −70°C or fixed immediately in 4% paraformaldehyde. Tissue sections were obtained either with a cryostat or a microtome after paraffin embedding. Fixed and paraffin embedded sections were then stained with hematoxylin and eosin according to standard protocols.

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