NTPs, 10 mM dithiothreitol, and 10 U ribonuclease H^- reverse transcriptase (SuperScript II RT, Life Technologies, Inc.) for 60 min at 42 C, 15 min at 50 C, and 15 min at 70 C. Subsequently, 1 μl of the resulting cDNA was used as a template for PCR. The resulting cDNA was used as a template for real-time PCR. Real-time PCR was carried out with the Light Cycler System (Roche, Mannheim, Germany) using the DNA binding dye Syber Green I (Roche) for the detection of PCR products. PCR was set up using 3 mM $MgCl_2$, 10 pmol/liter of each primer (Table 3), and 2.5 U *Taq* DNA polymerase (Life Technologies, Inc.). An initial denaturing step of 95 C for 1 min was followed by 40 cycles, respectively, of 95 C for 0 sec; 15-sec annealing at 58 C (PR-AB, PR-B, and AR), 60 C [ERβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], and 62 C (ERα); and extension for 15 sec at 72 C. The fluorescence intensity of the double-strand specific Syber Green I, which reflects the amount of specific PCR products formed, was read by the LightCycler at 85 C after the end of each extension step. After PCR, the products were resolved on a 2% agarose ethidium bromide gel. Images were captured with Polaroid (Hertfordshire, UK) film under UV light. In initial experiments PCR products were purified and subjected to direct sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Genetic Analyzer, PerkinElmer Corp., PE Applied Biosystems, Foster City, CA) to verify amplification of the correct sequences.

As a positive control, the MCF7 breast cancer cell line was used for ERα (23), and the T47D breast cancer cell line was used for ERβ (23), PR-AB (24), and PR-B (25). Testis was used as a positive control for AR. Negative control experiments lacked the cDNA substrate to check for the possibility of exogenous contaminating DNA. No amplified products were observed under these conditions.

The mRNA levels for ERα, ERβ, PR-AB, PR-B, and AR in each case are summarized as a ratio of GAPDH and evaluated as a ratio (percentage) compared with that of each positive control. Conventional quantitative PCR requires utilization of a purified plasma cDNA in the construction of a standard curve, but PCR products could be semiquantitated with the LightCycler using purified cDNA of known concentrations (26, 27).

Statistical analysis

Statistical analyses among patient age, tumor size, Ki-67 LI, H-scores, and steroid receptor mRNA levels were performed using a correlation coefficient (r) and a regression equation. Statistical differences between the H-score for steroid receptors and sex, status of myasthenia gravis, clinical stage, or WHO classification were evaluated using Mann-Whitney and Kruskal-Wallis tests (28). Overall survival curves were generated according to the Kaplan-Meier method (29), and statistical significance was calculated using the log-rank test (30). Univariate and multivariate analyses were evaluated by a proportional hazard model (COX) using PROC PHREG in our SAS software. *P* value less than 0.05 was considered significant.

Results

Immunohistochemical expression of sex steroid receptors

ERα, ERβ, PR-A, PR-B, and AR immunoreactivity was detected predominantly in the nuclei of epithelial cells of thymoma (Fig. 1, A and B), but not in lymphocytes, except for ERβ (Fig. 1, C and D). The number of positive cases and the H-score value (mean ± SD) for each receptor in 132 cases of thymoma were as follows: ERα, 87 (66%) and 85.8 ± 80.2; ERβ, 9 (7%) and 7.2 ± 8.7; PR-A, 5 (4%) and 2.7 ± 4.9; PR-B, 65 (49%) and 55.8 ± 68.3; and AR, 20 (15%) and 14.1 ± 11.7, respectively (Fig. 2A). There was a significant positive correlation between the immunoreactivity for ERα and PR-B ($r = 0.50$; $P < 0.0001$; Fig. 2B), but no other significant correlations were detected among the findings for sex steroid receptor immunoreactivity in this study.

Real-time PCR analysis

In the present study mRNA expression for ERα, ERβ, PR-AB, PR-B, AR, and GAPDH was detected as a specific single band at 168, 225, 196, 196, 195, and 307 bp, respectively (data not shown). The clinical data for the 20 patients included in our PCR analyses, including sex, age, tumor size, the presence or absence of myasthenia gravis, WHO classification, and clinical stage, are summarized in Table 1B.

A significant positive correlation was detected between the immunoreactivity (ir) for ERα and ERα mRNA levels ($r = 0.83$; $P < 0.0001$; Fig. 3A), irPR-B and PR-B mRNA levels ($r = 0.78$; $P < 0.0001$; Fig. 3B), or irAR and AR mRNA levels ($r = 0.72$; $P = 0.00030$). There were no significant positive correlations between the immunoreactivity for ERβ and ERβ mRNA levels ($r = 0.032$; $P = 0.21$), irPR-A and PR-AB mRNA levels ($r = 0.28$; $P = 0.13$), or irPR-B and PR-AB mRNA levels ($r = 0.33$; $P = 0.091$).

Correlation between immunoreactivity for sex steroid receptors and clinicopathological parameters

The association between ERα immunoreactivity and clinicopathological factors for thymomas is summarized in Table 4. There was an inverse correlation between ERα immunoreactivity and tumor size ($r = 0.28$; $P = 0.0010$), clinical stage ($P < 0.0001$), WHO classification ($P = 0.015$), and Ki-67 LI ($r = 0.43$; $P < 0.0001$; Fig. 4A). However, in this study there was no significant relationship between ERα immunoreactivity and sex, patient age, menopausal status, and/or status of myasthenia gravis.

The association between PR-B immunoreactivity and clinicopathological factors for thymomas is summarized in Table 4B. PR-B immunoreactivity was inversely correlated with

TABLE 3. Oligonucleotide primer sequences used for RT-PCR

mRNA	Sequence	cDNA position	bp	Source/GenBank accession no.
ERα	FWD 5′-AAG AGC TGC CAG GCC TGC C-3′ REV 5′-TTG GCA GCT CTC ATG TCT CC-3′	702–869	168	Ref. 21
ERβ	FWD 5′-GCT CAA TTC CAG TAT GTA CC-3′ REV 5′-GGA CCA CAT TTT TGC ACT-3′	1313–1537	225	AB 006590
PR-AB	FWD 5′-TGG AAG AAA TGA CTG CAT CG-3′ REV 5′-TAG GGC TTG GCT TTC ATT TG-3′	1987–2182	196	NM 000926
PR-B	FWD 5′-ACA CCT TGC CTG AAG TTT CG-3′ REV 5′-CTG TCC TTT TCT GGG GGA CT-3′	303–498	196	NM 000926
AR	FWD 5′-TAC CAG CTC ACC AAG CTC C T-3′ REV 5′-GCT TCA CTG GGT GTG GAA AT-3′	3097–3291	195	M 23263
GAPDH	FWD 5′-TGA ACG GGA AGC TCA CTG G-3′ REV 5′-TCC ACC ACC CTG TTG CTG TA-3′	731–1037	307	M 33197

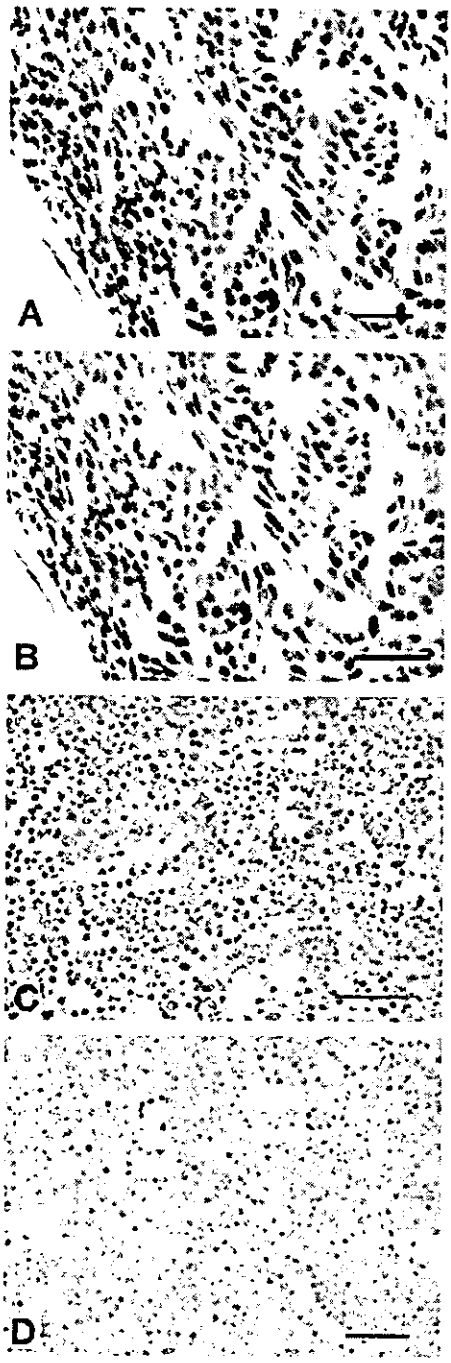

FIG. 1. Immunohistochemistry for ERα (A and C) and PR-B (B and D) in tissue specimens of human thymoma. Immunoreactivity for ERα and PR-B was detected in the nuclei of thymoma epithelial cells, but not in lymphocytes. A and B, Epithelial cell dominant type, which is type A by WHO classification (same field); C and D, lymphocyte dominant type, which is type B1 by WHO classification (same field). Original magnification, ×400. *Bar*, 25 μm.

clinical stage ($P = 0.00030$), WHO classification ($P = 0.028$), and Ki-67 LI ($r = 0.38$; $P < 0.0001$; Fig. 4B).

In the present study there was no significant correlations between immunoreactivity for other steroid receptors and clinicopathological parameters.

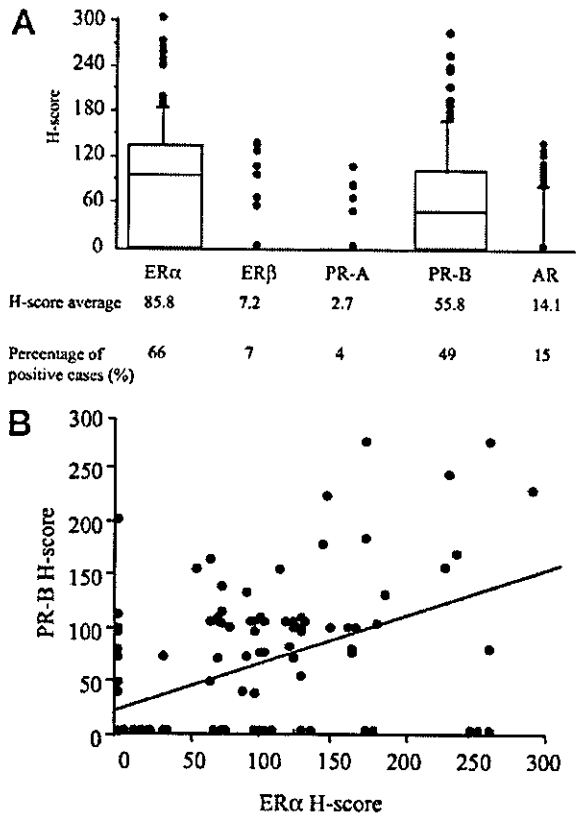

FIG. 2. A, Scattered plots showing variation in the H-score for ERα, ERβ, PR-A, PR-B, and AR in 132 human thymomas. The average H-score for each receptor and the percentage of positive cases are also shown. B, Correlation between the H-score for ERα and PR-B in 132 cases of thymoma. A significant correlation ($r = 0.50$; $P < 0.0001$) was detected. $y = 19.45 + 0.42x$; $r^2 = 0.25$.

Correlation between immunoreactivity for sex steroid receptors and overall survival of patients with thymoma

There was a significant positive correlation between ERα immunoreactivity and clinical outcome ($P = 0.00010$; Fig. 5A). No significant correlations were detected between the immunoreactivity for other steroid receptors and clinical outcome in patients with thymoma (Fig. 5B). In our female patients there was a significant positive correlation between ERα immunoreactivity and clinical outcome ($P = 0.00060$; Fig. 5D) and between PR-B immunoreactivity and clinical outcome ($P = 0.048$; Fig. 5F), but there were no significant correlations between ERα immunoreactivity ($P = 0.13$; Fig. 5C) or PR-B immunoreactivity ($P = 0.21$; Fig. 5E) and clinical outcome in male patients with thymoma. After univariate analysis (Table 5), clinical stage ($P = 0.0017$), ERα immunoreactivity ($P = 0.0021$), and tumor size ($P = 0.0024$) were demonstrated to be significant prognostic factors for overall survival in 132 thymoma patients. A multivariate analysis revealed that only clinical stage ($P = 0.024$) and ERα immunoreactivity ($P = 0.036$) were independent prognostic factors with relative risks over 1.0 in our series.

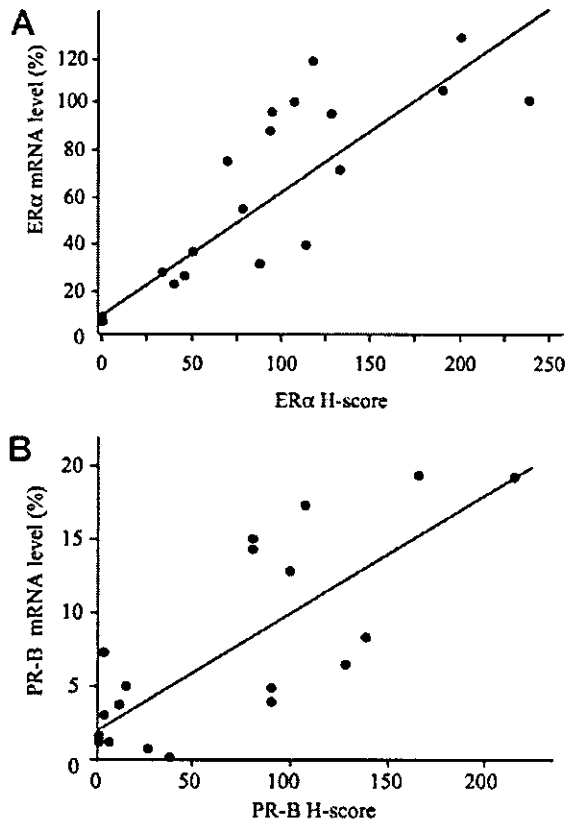

FIG. 3. Correlation between the H-score and the mRNA level of ERα or PR-B. Real-time PCR analysis was performed for ERα, ERβ, PR-A, PR-B, and AR in 20 cases of thymoma. A, Correlation between the H-score of ERα and mRNA level of ERα ($r = 0.83$; $P < 0.0001$; $y = 0.14 + 0.005x$; $r^2 = 0.70$). B, Correlation between the H-score of PR-B and the mRNA level of PR-B ($r = 0.78$; $P < 0.0001$; $y = 0.022 + 0.001x$; $r^2 = 0.61$).

Discussion

This is the first study demonstrating a correlation between the expression of sex steroid receptors and clinicopathological parameters in human thymoma. Various sex steroid receptors were found to be expressed predominantly in the epithelial cells of human thymoma. ERα immunoreactivity was detected in the great majority (66%) of human thymoma epithelial cells examined in our study, whereas ERβ immunoreactivity was detected in only 7% of the cases examined. Kawashima *et al.* (31) described the immunolocalization of ER and PR in epithelial cells of the rat thymus. A study using ERα knockout mice demonstrated that estrogen-ERα interaction was essential for the normal development and maturation of the thymus (9). In addition, estrogens have been suggested to regulate the production of thymosine α, a hormonal factor released from thymic epithelial cells (32). The relatively wide distribution of ERα immunoreactivity in human thymoma described in the present report appears to suggest the importance of estrogenic actions in human thymoma via ERα. Seiki and colleagues (33) demonstrated that estrogens could influence the T lymphocyte population in the thymus of ovariectomized female rats. However, in our study ERα immunoreactivity was not detected in lymphocytes, although ERβ was detected in lymphocytes in 10 cases (8%). Thymosine α, which is in part regulated by estrogen, is involved in immune modulations, including T lymphocyte differentiation (34). Recently, Tornwall *et al.* (35) reported that ERα was detected in all lymphocytes in the mouse thymus. Moreover, ERβ was shown to be expressed at low levels in thymic $CD4^-/CD8^-$ T cells in the mouse thymus (35).

TABLE 4A. Correlation between ERα immunoreactivity and pathological parameters in 132 human thymomas

	H-score of ER α	*P*	*r*
Patient age[a]		0.19	−0.12
Sex			
Male	75.1 ± 10.8		
Female	94.2 ± 9.1	0.097	
Premenopausal	95.9 ± 10.7		
Postmenopausal	92.3 ± 15.1	0.65	
Myasthenia gravis			
(+)	126.4 ± 23.7		
(−)	76.8 ± 6.5	0.069	
Tumor size (mm)[a]		0.0010	−0.28
Clinical stage			
I	114.4 ± 9.8		
II	84.1 ± 12.5		
III	43.3 ± 17.1	<0.0001	
IV	24.6 ± 10.8		
WHO classification			
A	111.1 ± 15.4		
AB	104.9 ± 16.2		
B1	96.3 ± 14.4	0.015	
B2	64.9 ± 12.6		
B3	36.5 ± 7.4		
Ki-67 LI[a]		<0.0001	−0.43
PR-B H-score[a]		<0.0001	0.50

[a] Data are presented as mean ± 95% confidence interval.
All other values represent mean ± SEM. $P < 0.05$ was considered significant.

TABLE 4B. Correlation between PR-B immunoreactivity and pathological parameters in 132 human thymomas

	H-score of PR-B	*P*	*r*
Patients age[a]		0.33	−0.086
Sex			
Male	58.7 ± 9.9		
Female	53.5 ± 7.3	0.92	
Premenopausal	51.9 ± 9.1		
Postmenopausal	55.2 ± 11.5	0.65	
Myasthenia gravis			
(+)	80.1 ± 18.7		
(−)	50.4 ± 5.9	0.25	
Tumor size (mm)[a]		0.026	−0.19
Clinical stage			
I	77.7 ± 8.6		
II	45.6 ± 11.9		
III	30.0 ± 14.1	0.00030	
IV	15.4 ± 8.5		
WHO classification			
A	85.7 ± 15.8		
AB	64.6 ± 14.0		
B1	51.3 ± 11.9	0.028	
B2	45.1 ± 9.8		
B3	18.0 ± 9.6		
Ki-67 LI[a]		<0.0001	−0.38

[a] Data are presented as mean ± 95% confidence interval.
All other values represent mean ± SEM. $P < 0.05$ was considered significant.

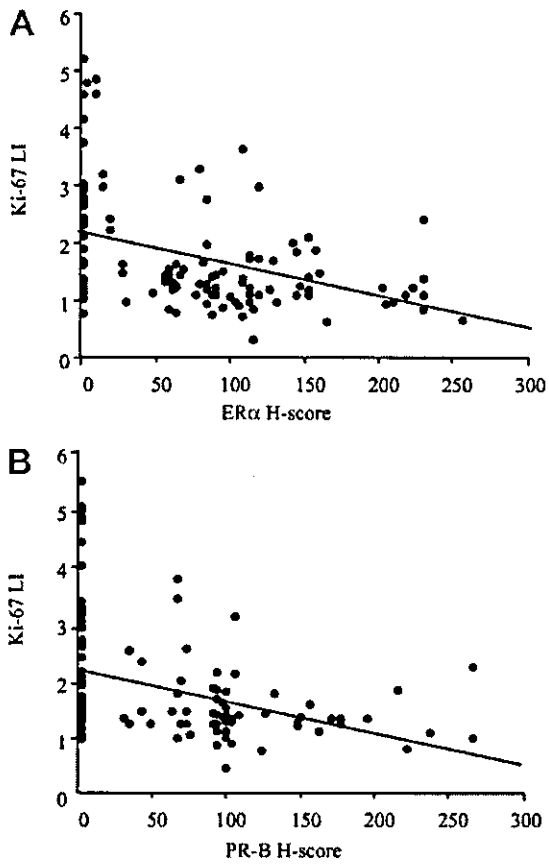

FIG. 4. Correlation between the H-score for ERα (A) or PR-B (B) and Ki-67 LI in 132 cases of thymoma. ERα and PR-B immunoreactivity was inversely correlated with Ki-67 LI (for ERα: $r = 0.43$; $P < 0.0001$; for PR-B: $r = 0.38$; $P < 0.0001$; for ERα: $y = 2.21 + 0.005x$; $r^2 = 0.18$; for PR-B: $y = 2.07 + 0.005x$; $r^2 = 0.14$).

Carbone *et al.* (36) examined the estrogen binding capacity of peripheral blood mononuclear cells and lymphocytes from two young female patients with myasthenia gravis and reported high levels of estrogen-binding sites in both prethymectomy peripheral blood mononuclear cells and lymphocytes from the hyperplastic thymus of myasthenia gravis patients. Therefore, differences from the results of our present study may represent differences between human and mouse species or between the neoplastic and nonneoplastic thymus. It is not clear at present what causes these changes; therefore, further investigations are required to clarify these discrepancies.

In the present study ERα immunoreactivity was inversely correlated with Ki-67 LI ($P < 0.0001$) and tumor size ($P = 0.0010$). Using the WHO classification, ERα-positive thymomas were found to be expressed at earlier clinical stages ($P < 0.0001$) with lower histological grades ($P = 0.015$). Moreover, patients with ERα-positive thymoma showed significantly better clinical outcome than those with ERα-negative thymoma ($P = 0.00010$). In addition to clinical stage, a well established diagnostic modality in human thymoma (14), the results of both univariate and multivariate analysis in our study demonstrated that ERα immunoreactivity is an independent prognostic factor ($P = 0.0021$ and $P = 0.036$, respectively) for human thymoma. Estrogen is well known to exert a wide variety of effects in target organs, including cell proliferation in breast cancer (12) and inhibition of cell growth in prostatic cancer (37) or vascular smooth muscle cells (38). In a study by Ezaki and co-workers (39), thymomas in males were reported to be larger than those of females in age-matched BUF-Mna rats. Furthermore, Ezaki's study (39) indicated that estrogen inhibited or retarded the development of spontaneous thymomas in BUF/Mna rats. The results from our present study are in good agreement with these published findings. These results all appear to suggest that estrogen may inhibit cell proliferation in human thymoma. ERα-positive thymoma may also be associated with increased estrogenic actions resulting in an improved prognosis.

On the other hand, there was a significant positive correlation between the immunoreactivity for ERα and PR-B ($r = 0.50$; $P < 0.0001$). PR, which is coexpressed with ER, has been regarded as one of the markers of functional estrogen pathways, *i.e.* transcription of the PR-B gene is enhanced and maintained by estrogens (12, 40). Therefore, PR-B status in human thymoma may also reflect estrogenic actions in human thymoma tissues, but other possibilities also exist concerning the biological significance of PR-B status in human thymoma. We examined survival data from patients in the present study according to the status of ERα and PR-B, separately in male and female patients diagnosed with thymoma. There was a significant positive correlation between ERα immunoreactivity and clinical outcome ($P = 0.0018$; Fig. 5D) and between PR-B immunoreactivity and clinical outcome ($P = 0.048$; Fig. 5F) in female patients, but no such significant correlations were detected in male patients with thymoma. These findings also suggest that the expression of ERα may represent a marker associated with better clinical outcome, especially in female patients with thymoma. PR-B status in human thymoma may also reflect estrogenic actions in thymoma tissues, but further investigations are required to demonstrate the presence of direct estrogenic actions in human thymoma.

The combination ERα analysis in resected surgical pathology specimens by immunohistochemistry and recently developed selective ER modulators has been considered for the diagnosis and treatment, respectively, of patients with advanced thymoma, especially females. However, further investigations are required to clarify the role of ER in the diagnosis, prevention of disease progression, and/or pathogenesis.

In this study immunoreactivity for PR-B was dominant in human thymomas (49%) compared with that for PR-A (4%). To date, two different isoforms of PR, PR-A (81–83 kDa) and PR-B (116–120 kDa), have been identified (4). The exact roles and functions of each receptor isoform remain unclear, but PR-B is believed to be necessary for the activation of progesterone target genes (4), whereas PR-A functions as a repressor of PR-B function (41). Transcription of the PR-B gene is enhanced and maintained by estrogen. PR, which is coexpressed with ER, has been regarded as one of the markers of functional estrogen pathways (12, 40). Therefore, PR-B status in human thymoma may also reflect estrogenic actions

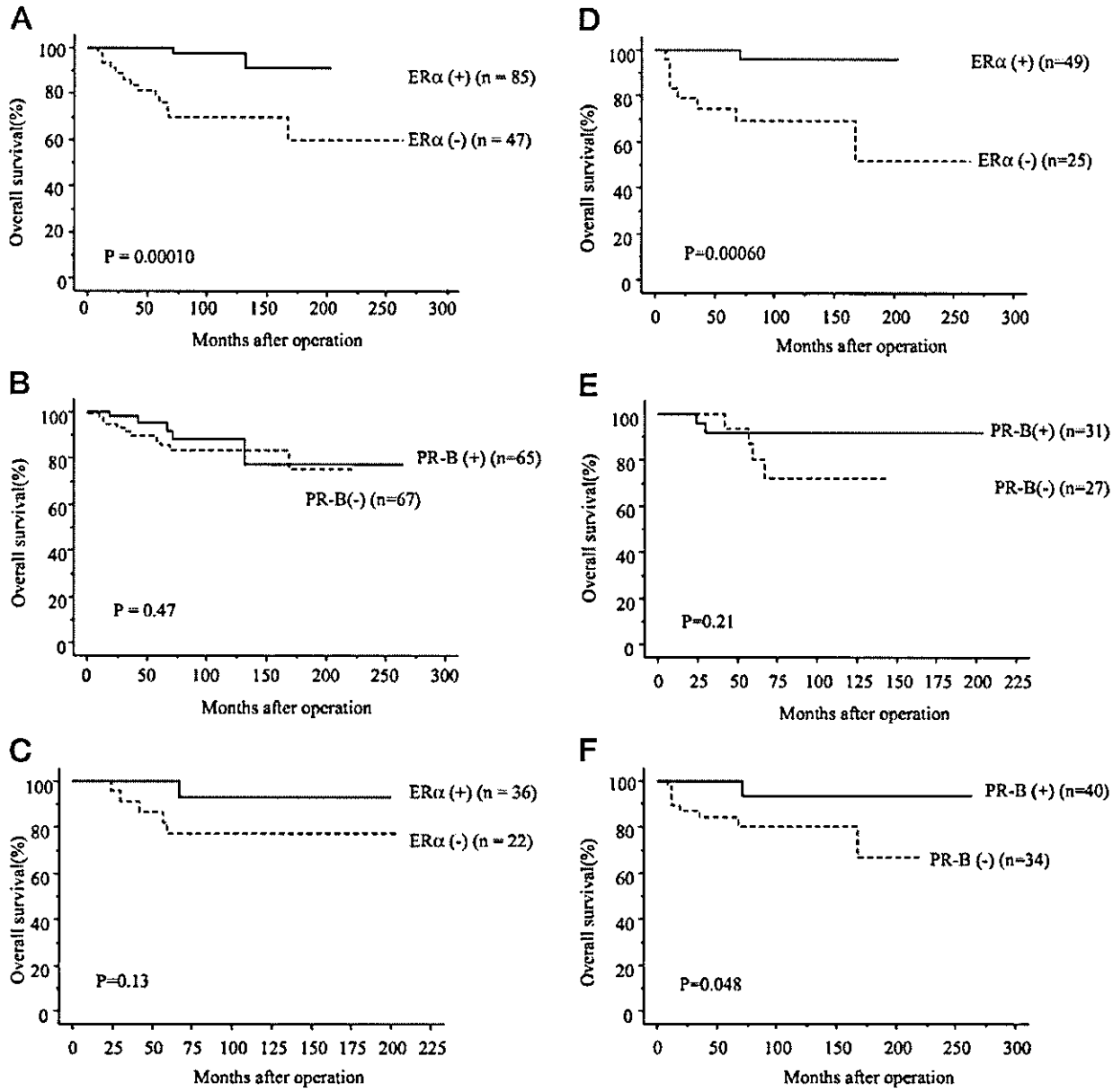

FIG. 5. Overall survival of 132 patients with thymoma with respect to ERα (A) or PR-B (B) immunoreactivity (Kaplan-Meier method). ERα immunoreactivity was significantly associated with an improved overall survival ($P = 0.00010$); however, PR-B immunoreactivity was not significantly associated with overall survival ($P = 0.47$). There was a significant positive correlation between ERα immunoreactivity and clinical outcome ($P = 0.00060$; Fig. 5D) in female patients, but no significant correlations in male patients ($P = 0.13$; Fig. 5C). Furthermore, a significant positive correlation between PR-B immunoreactivity and clinical outcome ($P = 0.048$; Fig. 5F) was found in female patients, but no significant correlations were identified in male patients ($P = 0.21$; Fig. 5E).

in human thymoma tissues. However, further investigations are required to clarify the role of PR-B in human thymoma.

In this study AR immunoreactivity was detected in only 15% of human thymomas, and the status of AR was by no means significantly correlated with any clinicopathological parameters examined in this study. Androgenic action in the thymus is still very controversial. Castration of normal male rodents results in significant enlargement of the thymus (42). Furthermore, a study by Nancy *et al.* (42) reported that AR immunolocalization in the thymic epithelium and androgens modulated the size of the thymus and thymocyte development in the male mouse. Dulos *et al.* (43) found that indirect actions of androgen could accelerate the apoptosis of thymocytes in the mouse model. However, Ezaki *et al.* (39) reported that androgens were not involved in spontaneous thymoma tumor development in BUF/Mna rats. In this study we did not investigate the role of androgens in human thymoma tissues. Further studies are required to clarify the

TABLE 5. Univariate and multivariate analyses of overall survival in 132 thymoma patients examined

Variable	Univariate	Multivariate	
	P	*P*	Relative risk (95% CI)
Clinical stage (II, III, IV/I)	0.0017	0.024	5.78 (1.71–47.20)
ERα (−/+)	0.0021	0.036	4.79 (1.14–21.96)
Tumor size (>60mm/ ≥60mm)	0.0024		
Female (premenoposal/ postmenoposal)	0.062		
PR-B (−/+)	0.47		
Sex (male/female)	0.70		

CI, Confidence interval.

role that androgens play in the pathogenesis of human thymoma.

In summary, we have characterized the expression of various sex steroid receptors in human thymoma. We found ERα and PR-B immunoreactivity to be widely distributed in thymoma epithelial cells. ERα and PR-B immunoreactivity was inversely associated with tumor size, clinical stage, WHO classification, and Ki-67 LI; moreover, ERα was found to be an independent prognostic variable in multivariate analysis. Furthermore, the results from our present study suggest that estrogen inhibits the growth of thymoma via ERα, and that ERα immunoreactivity is a potent prognostic factor in human thymoma.

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Retinoid Receptors in the Human Endometrium and Its Disorders: A Possible Modulator of 17β-Hydroxysteroid Dehydrogenase*

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ABSTRACT

Retinoids have recently been proposed to modulate estrogenic actions in various sex steroid-dependent neoplasms, but little has been studied in human endometrial disorders. Therefore, in this study, we first examined the immunolocalization of retinoic acid receptor α, β, and γ, and retinoid X receptor (RXR) α, β, and γ in 20 normal cycling human endometria, 34 endometrial hyperplasia, and 46 endometrioid endometrial adenocarcinomas. We then correlated these findings with other clinicopathological parameters, especially in the correlation between retinoid receptor subtypes and the status of steroid hormone receptors, 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase. We also then examined the effects of retinoic acid on the expression of 17β-HSD type 2 in cell lines derived from endometrial carcinoma using Northern blotting analysis to examine the possible roles of retinoids in *in situ* endometrial estrogen metabolism. Among these six retinoid receptors examined, RXRγ immunoreactivity was exclusively detected in the epithelial cells of the secretory phase endometrium but not of the proliferative phase, which was well correlated with 17β-HSD type 2 immunolocalization. However, in endometrial hyperplasia, RXRγ was not correlated with 17β-HSD type 2. In endometrioid endometrial adenocarcinoma, there was a statistically significant correlation between 17β-HSD type 2 immunoreactivity and RXRγ labeling index (LI) ($P < 0.001$) and between RXRγ LI and progesterone receptor LI ($r = 0.501$, $P = 0.003$).

A significant inverse correlation was also detected between RXRγ LI and patient age ($r = 0.449$, $P = 0.015$). No statistically significant correlation was obtained between LIs of receptors and other clinicopathological parameters including the status of intratumoral aromatase examined by immunohistochemistry. In the endometrial carcinoma cell line, RL95–2, retinoic acid markedly increased the level of 17β-HSD type 2 messenger RNA in a time- and dose-dependent manner. These results all suggest that retinoic acids may be involved in modulation of *in situ* estrogen metabolism in both normal and neoplastic human endometrium possibly through RXRγ by stimulating the expression of 17β-HSD type 2. (*J Clin Endocrinol Metab* 86: 2721–2727, 2001)

ENDOMETRIAL CARCINOMA IS one of the most common female pelvic malignancies in the world, and its incidence has recently increased (1, 2). Previous clinical, biological, and epidemiological findings all suggest that prolonged or unopposed estrogenic stimulation increased the risk of endometrial carcinoma, especially of the endometrioid type (3, 4). However, there has been no consistent evidence of increased serum estrogen concentrations in women with endometrial disorders (5–7). Recently, *in situ* estrogen metabolism and synthesis have been considered to play a very important role in the development and progression of various human steroid hormone-dependent epithelial neoplasms, including endometrial carcinoma (8). It therefore becomes very important to study the expression and/or regulation of the enzymes involved in *in situ* estrogen metabolism in human endometrial malignancy to understand its local hormonal involvement. Among these enzymes, aromatase, which catalyzes the conversion of androgens to estrogens, and 17β hydroxysteroid dehydrogenase (17β-HSD) isozymes, which catalyze the interconversion of E_2 and estrone (E_1), are two principal enzymes involved in the formation (9–11).

Retinoids, metabolites of vitamin A, have been recently demonstrated to play very important roles in *in situ* estrogen metabolism through the regulation of steroid hormone receptors and17β-HSD. The possible therapeutic implications related to this property have been proposed in human breast cancer (12–16). Retinoids are known to exert their effects through their binding to specific receptors and subsequent modulation of specific gene expression in their target tissues. Roma *et al.* (13) reported that estrogen receptor (ER)-positive breast cancer cell lines express significantly higher levels of retinoic acid receptors (RAR) α than ER-negative cell lines. They also reported a significant correlation between RARα and ERα in human breast cancer tissues (13). Reed *et al.* (14) also reported that retinoic acid increases the expression of 17β-HSD type 1 messenger RNA (mRNA) and reductive activity in breast cancer cell lines. Suzuki *et al.* (15) reported a significant correlation between RARα and 17β-HSD type 1 expression in human breast carcinoma. These data all suggest that retinoids may modulate *in situ* estrogen metabolism

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through their receptors in estrogen receptor-positive human breast carcinomas, possibly through RARα.

In the normal human endometrium, intracellular retinoic acid concentrations in both epithelial and stromal cells are elevated during the secretory phase (17). Kumarendran *et al.* (18) also reported the presence of the mRNA expression of RARα, RARβ, RARγ, and RXR (retinoid X receptor) α using Northern blotting in normal human endometrium. Siddiqui *et al.* (19) then reported the presence of RAR and RXR mRNA using Northern blotting in endometrioid endometrial carcinoma. However, the details of the status of these retinoid receptors and the correlation between retinoid receptors and local estrogenic metabolism has not been studied in the human endometrium and its disorders.

Therefore, in this study we first examined the cellular localization of RARs and RXRs using immunohistochemistry. We then correlated these findings with clinicopathological parameters, including the status of steroid hormone receptors, 17β-HSD type 2 and aromatase, in the human endometrium and its disorders to study the possible roles and regulatory mechanism of retinoids in *in situ* estrogen metabolism. Using endometrial cancer cell lines, we also examined the effects of retinoids on 17β-HSD type 2 mRNA expression for further analysis.

Materials and Methods

Patients and tissues

Twenty normal-cycling human endometria (41 ± 3.7 yr old), 33 endometrial hyperplasias (42 ± 9.2 yr old), and 46 endometrial endometrioid adenocarcinomas (58 ± 9.8 yr old; grade 1: 23 cases; grade 2: 14 cases; grade 3: 9 cases) were retrieved from the surgical pathology files of Tohoku University Hospital, Sendai, Japan. None of these patients had received preoperative chemotherapy or pelvic radiation. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by the World Health Organization and staged according to the International Federation of Gynecology and Obstetrics system (20, 21). All specimens were routinely processed (*i.e.*, 10% formalin fixed for 24–48 h), paraffin embedded, and thin sectioned (3 μm).

Antibodies

Polyclonal antibodies for RARα (sc-551), RARβ (sc-552), RARγ (sc550) were purchased (Santa Cruz Biotechnology, Santa Cruz, CA). Polyclonal antibodies for RXRα, RXRβ, and RXRγ were raised against synthetic peptides containing the following mouse RXR amino acid residues: RXRα 92–109; RXRβ 78–93; RXRγ 35–54. The characterization of the RXR antibodies was confirmed by Western blotting and immunoprecipitation as described previously (22), and utilization of these antibodies for immunohistochemistry was also reported previously (23).

The monoclonal antibody of 17β-HSD type 2, mAB-C2–12, was produced by immunizing mice with a synthetic carboxyl-terminal peptide corresponding to amino acids 375–387 of 17β-HSD type 2 and was provided by Dr. S. Andersson, University of Texas Southwestern Medical Center, Dallas, TX (24). Aromatase antibody was prepared against enzyme purified from human placenta and was provided by Dr. N. Harada, Fujita-Gakuen Health University, Toyoake, Japan (25).

The monoclonal antibody for progesterone receptor (PR) (Chemicon, Temecula, CA) and the monoclonal antibody for ERα (Immunotech, Marseille, France) were used.

Immunohistochemistry

Immunohistochemical analysis was performed employing the streptavidin-biotin amplification method using a Histofine kit (Nichirei, Tokyo, Japan) and has been previously described in detail (26). Tissue sections of full-term placenta were used as positive controls for 17β-HSD type 2. Human breast carcinoma was used as a positive control for retinoid and steroid receptors. As a negative control, normal rabbit or mouse IgG was used instead of the primary antibodies. In RXRs, immunohistochemical preabsorption tests were also performed. No specific immunoreactivity was detected in these tissue sections.

Semiquantitative analysis of immunohistochemical staining

In retinoid receptors, ERs, and PRs, more than 500 glandular or carcinoma cells were counted in each case by two of the authors (K.I. and T.S.) independently, after reviewing the slides and determining the areas of evaluation simultaneously, using a double-headed microscope. The percentage of immunoreactivity (*i.e.*, labeling index [LI]) was subsequently determined. Cases with interobserver differences of more than 5%, which occurred in 3–7% of the cases examined were re-evaluated together using double-headed microscopy. Intraobserver differences were less than 5% when examining the same selected fields of representative cases. The mean value was obtained in cases with interobserver differences of less than 5%. For 17β-HSD type 2 immunostaining, glandular or carcinoma cells were divided into the following three groups: 2+: more than 50% positive cells, +: 0–50% positive cells; −: no immunoreactivity, based on the report by Sasano *et al.* (23). For aromatase immunostaining, the findings were also classified as follows according to Watanabe *et al.* (9). The aromatase-positive stromal cells were divided into the following three groups: 1), 0–5%; 2), 5–25%; and 3) more than 25% of cells positive for aromatase. Evaluation of immunoreactivity of these steroidogenic enzymes was performed in the same manner as that of the nuclear antigen described above.

Statistical analysis

Statistical analyses among LIs of retinoid receptors, ERs, PRs, and KI-67, and patient age were performed by the correlation coefficient (r) and the regression equation. Association between LIs of retinoid receptors and 17β-HSD immunoreactivity, stage, and histological grade were all evaluated using a Bonferroni test. *P* values less than 0.05 were considered significant. We corrected the data with a Bonfferoni post hoc test, and multiple correlations were performed among clinicopathological parameters.

Cell culture

We examined five human endometrial cancer cell lines in this study: RL95–2, HEC-1A, HEC1-B, and KLE, obtained from the American Type Culture Collection (Manassas, VA), and the Ishikawa cell line provided by Dr. Sakurada (Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Sendai, Japan). The cells were cultured with Ham F12: DMEM (1:1, vol/vol) containing 10% FBS and passed at confluence to plastic culture dishes (100-mm diameter; Becton Dickinson and Co. Lincoln Park, NJ) for 17β-HSD type 2 mRNA analyses.

RNA extraction and Northern blot analysis

Total cellular RNA was extracted with lithium chlolide/urea from cells grown in monolayers according to the method of Chirgwin *et al.* (27). Total RNA (10 μg/lane) was size fractionated by electrophoresis on formaldehyde-agarose (1%) gels and transferred electrophoretically to a nylon membrane. A P-32–labeled 17β-HSD type 2 complementary DNA was employed as a probe provided by Dr. S. Anderson (University of Texas Southwestern Medical Center, Dallas, TX). An oligonucleotide probe of glyceraldehyde-3-phosphate dehydrogenase (G3PDH; 24-mer) was used as an internal standard. Specific radioactivity was assayed by AMBIS radioanalytic imaging system (AMBIS Inc., San Diego, CA). The mRNA level of 17β-HSD type 2 was evaluated as the ratio of the radiointensity, compared with that of G3PDH and is expressed as percent change, compared with the control.

Total RNA (10 μg/lane) of nontreated RL95–2, HEC-1A, HEC-1B, KLE, and Ishikawa cells was evaluated by Northern analyses for 17β-HSD type 2. Next, for checking the effects of RA derivatives and ligands for RA receptors on the level of 17β-HSD type 2 mRNA in RL95–2 cells, cells were treated with three RA derivatives (t-RA, 13-*cis*-RA, and 9-*cis*-RA) and two RA receptor selective RA agonists (TTNPB for RAR and LG69 for RXR, 1 μM for each) for 32 h.

For evaluating the dose response to t-RA and 13-*cis*-RA on the levels of 17β-HSD type 2 mRNA in RL95–2 cells, the cells were treated with t-RA (closed bars) or 13-*cis*-RA (open bar) in six different concentrations (0, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM) for 32 h. The levels of 17β-HSD type 2 mRNA were evaluated as percent change, compared with the control (nontreatment for 32 h). Then for confirming time course of t-RA action on the level of 17β-HSD type 2 mRNA in RL95–2 cells, the cells were treated with (closed circles) or without (open circles) t-RA (1 μM) for 0, 2, 4, 8, 16, 32, and 64 h. The levels of 17β-HSD type 2 mRNA were evaluated as percent change, compared with those of nontreated cells.

Results

Normal cycling endometrium

RARα,β,γ and RXRα,β,γ immunoreactivities were all detected in the nuclei of some endometrial stromal cells throughout the phases of the menstrual cycle. RARα and RXRα,γ immunoreactivities were detected in the nuclei of 60–70% of stromal cells, whereas RARβ,γ and RXRβ immunoreactivities were present in the nuclei of 3–5% of stromal cells throughout the phases of the menstrual cycle. RARγ and RXRβ immunoreactivity was not detected in any of the epithelial cells examined. RARα, RARβ, and RXRα immunoreactivity was detected in the nuclei of epithelial cells throughout all menstrual phases. RXRγ immunoreactivity was detected in the nuclei of epithelial cells of the secretory phase endometrium but not of the proliferative phase (Fig. 1, A and B).

17β-HSD type 2 immunoreactivity was detected only in the cytoplasm of epithelial cells of the secretory phase endometrium but not of the proliferative phase. Aromatase immunoreactivity was undetected in all of the cases examined. ER and PR immunoreactivities were detected in the nuclei of epithelial and stromal cells through all phases.

Endometrial hyperplasia

Results are summarized in Table 1. Immunoreactivity for retinoid receptors was detected in the nuclei of both epithelial and stromal cells. RARα,β,γ and RXRα,β,γ immunoreactivity was detected in all the nuclei of some stromal cells in all the cases examined. RARα and RXRα,γ immunoreactivities were detected in the nuclei of 30–50% of stromal cells, whereas RARβ,γ and RXRβ immunoreactivities were present in the nuclei of 3–5% of stromal cells. RARα, RARβ, RXRα, and RXRγ immunoreactivity was detected in all the nuclei of hyperplastic glands. 17β-HSD type 2 immunoreactivity was detected in 24/33 cases (72.7%). 17β-HSD type 2 immunoreactivity tended to be correlated with RXRγ LI, but the correlation did not reach statistical significance ($P = 0.2$). No significant correlation was detected between retinoid receptor immunoreactivity and ER LI; PR LI; age; or histological classification including simple, complex, and atypical endometrial hyperplasia (data not shown). Aromatase immunoreactivity was undetected in all of the cases examined.

Endometrial carcinoma

Results are summarized in Table 1. RAR and RXR immunoreactivity was not detected in stromal cells in all the cases examined. RARα, RARβ, RXRα, and RXRγ immunoreactivity was detected in the nuclei of carcinoma cells (Fig. 2A). Marked aromatase immunoreactivity was detected in stromal cells in 50% of the cases of endometrial carcinoma, especially at the sites of frank invasion.

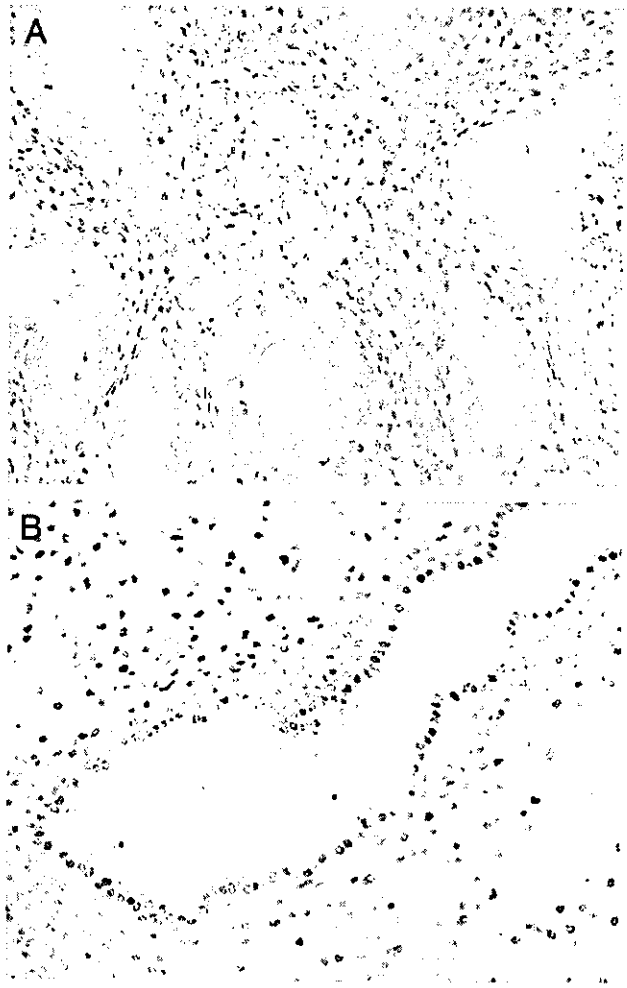

FIG. 1. Immunohistochemistry for RXRγ. A, Proliferative phase. B, Secretory phase. RXRγ immunoreactivity was detected in the nuclei of epithelial cells of the secretory phase endometrium but not of the proliferative phase. Original magnification, ×200.

17β-HSD type 2 immunoreactivity was detected in 37% (17/46) of the cases of endometrial carcinoma. In serial tissue sections, the great majority of RXRγ immunopositive carcinoma cells were also positive for 17β-HSD type 2 (Fig. 2, A and B). In addition, a statistically significant correlation was detected between 17β-HSD type 2 immunoreactivity and RXRγ LI ($P < 0.001$) (Fig. 3). A significant positive correlation was also detected between RXRγ LI and PR LI ($r = 0.501$, $P = 0.003$) (Fig. 4). A significant inverse correlation was also detected between RXRγ LI and patient age ($r = 0.449$, $P = 0.015$). No statistically significant correlation was obtained between LIs of any other retinoid receptor subtypes and other clinicopathological parameters, including the status of

TABLE 1. Summary of various receptor subtypes in endometrial hyperplasia and carcinoma cases

	LI mean (%)					
	RARα	RARβ	RXRα	RXRγ	ER	PR
Hyperplasia						
Simple	56.0 ± 21.4	14.2 ± 21.6	58.1 ± 20.2	12.1 ± 19.3	48.1 ± 10.6	46.2 ± 8.1
Complex	68.2 ± 23.0	7.0 ± 22.1	68.1 ± 18.2	23.0 ± 25.7	43.3 ± 8.3	50.8 ± 6.9
Atypical	58.1 ± 27.2	0.0	56.0 ± 28.1	1.7 ± 4.1	50.6 ± 9.8	47.0 ± 12.1
Total	60.5 ± 25.0	9.4 ± 20.0	60.1 ± 20.1	13.5 ± 20.8	47.1 ± 9.9	47.8 ± 8.7
Carcinoma						
Grade 1	65.5 ± 26.0	21.5 ± 26.2	58.0 ± 26.0	20.9 ± 27.8	45.2 ± 22.5	36.3 ± 24.6
Grade 2	64.1 ± 26.2	14.1 ± 20.2	46.2 ± 24.2	17.6 ± 25.8	46.1 ± 17.6	26.9 ± 17.8
Grade 3	52.1 ± 29.3	11.7 ± 17.0	58.3 ± 14.0	9.9 ± 18.5	19.7 ± 17.6	35.8 ± 15.8
Total	62.0 ± 28.1	17.3 ± 22.9	56.0 ± 23.0	17.7 ± 25.5	40.5 ± 22.3	33.3 ± 22.9

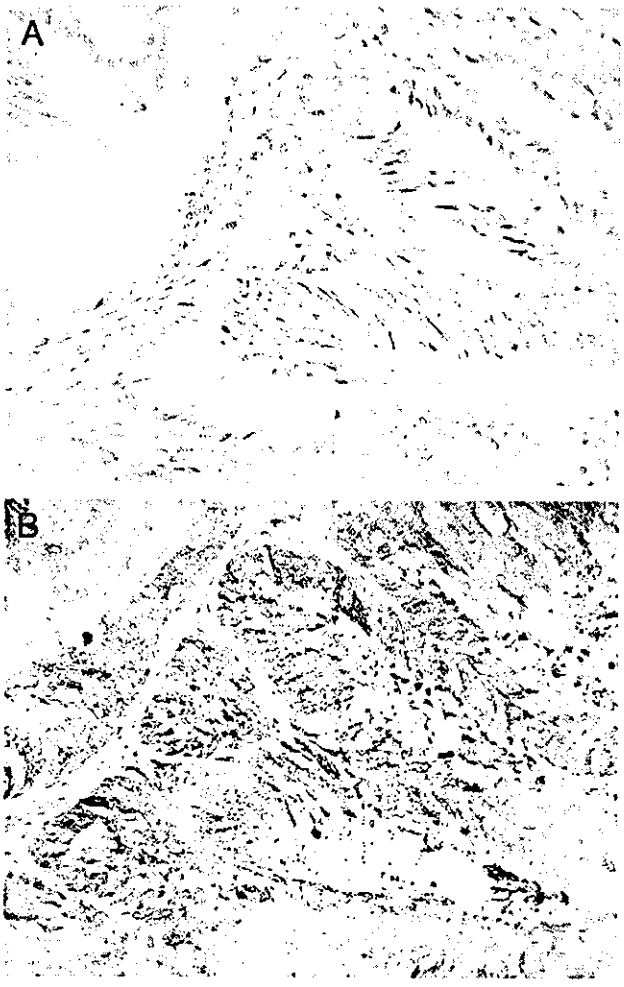

FIG. 2. Immunohistochemistry for RXRγ (A) and 17β-HSD type 2 (B) in human endometrioid endometrial adenocarcinoma in serial tissue sections. The great majority of RXRγ immunopositive cancer cells were also positive for 17β-HSD type2. Original magnification, ×200.

aromatase immunoreactivity, ER LI, clinical stage, and histological grade (data not shown).

There is no statistically significant correlation between LIs of retinoid receptor subtypes in epithelial cells and in stromal cells (data not shown).

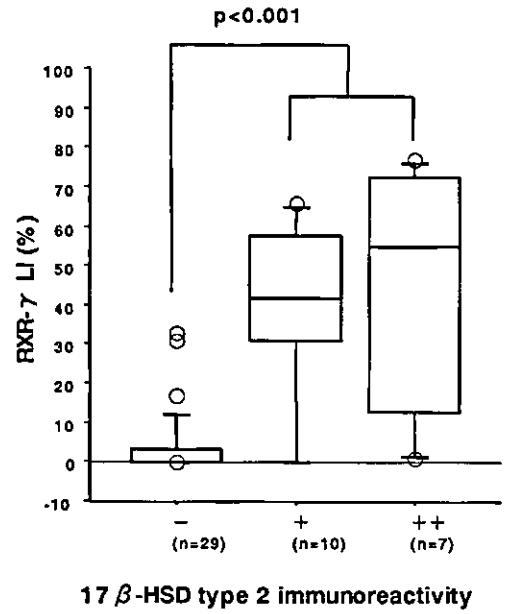

FIG. 3. Correlation between the immunoreactivity for 17β-HSD type 2 and RXRγ LI in human endometrioid endometrial adenocarcinoma. There was a statistically significant correlation between 17β-HSD type 2 immunoreactivity and RXRγ LI ($P < 0.001$). Data are presented as means ± 95% confidence interval.

Cell culture

17β-HSD type 2 mRNA was detected in RL95–2 and HEC-1A cells but not in the other endometrial cancer cell line. The level of 17β-HSD type 2 mRNA, as detected by densitometry in Northern blots, in RL95–2 cells was approximately 100 times more than that of HEC-1A cell. We therefore evaluated the effects of RA on the level of 17β-HSD type 2 mRNA in RL95–2 cells. Results of the effects of various RA derivatives and ligands for RA receptors employed in this study on the level of 17β-HSD type 2 mRNA in RL95–2 cells are shown in Fig. 5A. The relative level of induction of 17β-HSD type 2 mRNA, compared with controls, determined by densitometry was as follows: t-RA: 7.1, 13-*cis*-RA: 9.2, 9-*cis*-RA: 9.1, TTNPB: 7.9, LG69: 3.0 (Fig. 5B). Dose-response experiments on the level of 17β-HSD type 2 mRNA by t-RA and 13-*cis*-RA in RL95–2 cells revealed that the level of 17β-HSD type 2 mRNA was increased in a dose-dependent manner by t-RA and 13-*cis*-RA (Fig. 6A). Time course of t-RA on the level

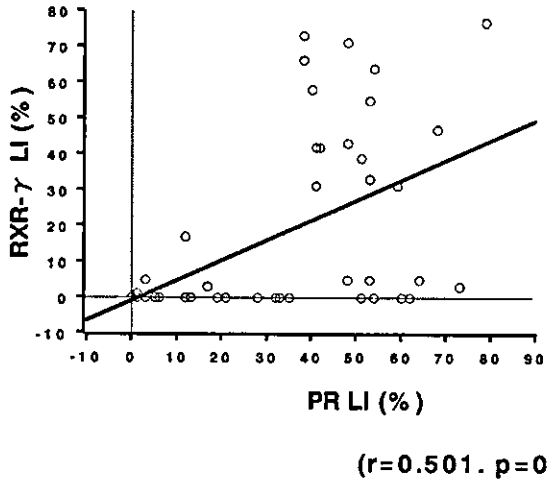

FIG. 4. Correlation between RXRγ LI and PR LI in human endometrioid endometrial adenocarcinoma. A significant correlation was detected between RXRγ LI and PR LI (r = 0.501, P = 0.003).

of 17β-HSD type 2 mRNA is also summarized in Fig. 6B. T-R-induced 17β-HSD type 2 mRNA in a time-dependent manner for up to 32 h. After 32 h of treatment, the level of 17β-HSD type 2 mRNA was still 4.9-fold greater than that of non-treated cells.

Discussion

In this study, we examined the localization of all six subtypes of retinoid receptors in human endometrium and its disorders. Among these receptor subtypes, RARα, RARβ, RXRα, and RXRγ were widely distributed, compared with other retinoid receptor subtypes, in both neoplastic and non-neoplastic human endometrium. Especially, immunoreactivity for RARα, RARβ, and RXRα was widely distributed in both epithelial and stromal cells of endometrial tissues throughout the menstrual cycle. The fact that these receptors were present in the stromal cells of normal endometrium and hyperplasia but not in endometrial carcinoma suggests possible alterations of characteristics of stromal cells and/or fibroblasts because of stromal invasion. We then examined the possible correlation between patterns of retinoid receptor immunolocalization and *in situ* estrogen metabolism and actions. In normal endometrium, Casey *et al.* (28) and Zeitoun *et al.* (29) reported that 17β-HSD type 2 mRNA was markedly expressed in endometrial glandural epithelial cells during the secretory phase but not during the proliferative phase. Mustonen *et al.* (30) also reported the same results using mRNA *in situ* hybridization. On the other hand, 17β-HSD type 1 mRNA was reported to be expressed at very low levels, compared with 17β-HSD type 2 mRNA in normal endometrium (31). In our study, 17β-HSD type 2 immunoreactivity was also detected only in the cytoplasm of epithelial cells in the secretory phase endometrium and not in the proliferative phase. Intracellular retinoic acid concentrations in both epithelial and stromal cells of endometrial mucosa are considered to be elevated during the secretory phase because of a marked reduction of cellular retinoic acid-binding protein type II mRNA (17). In normal endometrium, RXRγ was

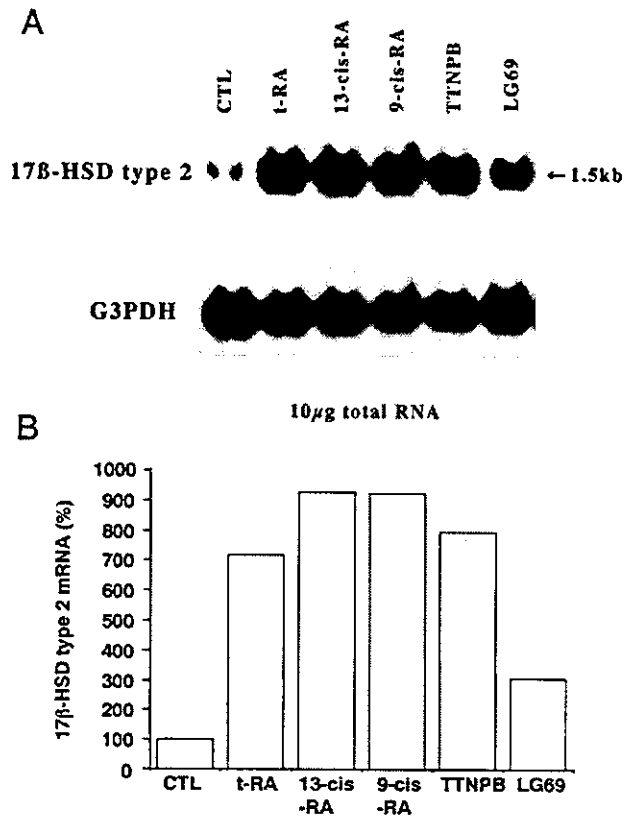

FIG. 5. A, Northern blot analysis demonstrating the effects of RA derivatives and ligands for RA receptors on the level of 17β-HSD type 2 mRNA in RL95–2 cells. Cells were treated with three RA derivatives (t-RA, 13-*cis*-RA, and 9-*cis*-RA) and two RA receptor selective RA agonists (TTNPB for RAR and LG69 for RXR, 1 μM for each) for 32 h. B, The relative level of induction of 17β-HSD type 2 mRNA, compared with controls, determined by densitometry was as follows: t-RA: 7.1, 13-*cis*-RA: 9.2, 9-*cis*-RA: 9.1, TTNPB: 7.9, LG69: 3.0.

detected only in the secretory phase among retinoid receptor subtypes examined. These results all indicated that increased retinoic acid concentrations are considered to exert their effects on secretory phase mucosa, possibly through RXRγ.

We therefore further characterized the possible correlation between retinoids and their receptors and 17β-HSD type 2 in endometrial disorders. Among retinoid receptor subtypes, the status of RXRγ tended to be correlated with 17β-HSD type 2 in endometrial hyperplasia, although not statistically significant. A significant positive correlation was, however, detected between RXRγ LI and 17β-HSD type 2 immunoreactivity ($P < 0.001$) in endometrial carcinoma. There were no correlations between the status of retinoid receptor subtypes and aromatase and/or estrogen receptor status in patients diagnosed with endometrial cancer. These findings suggest that among the factors that influence *in situ* estrogen metabolism and actions, the status of RXRγ was correlated with that of 17β-HSD type 2 at least in normal and neoplastic endometrium. These results also suggest that the possible effects of retinoids on *in situ* steroid metabolism in the human endometrium and its disorders may be mediated through modulation of 17β-HSD type 2 expression and/or activity.

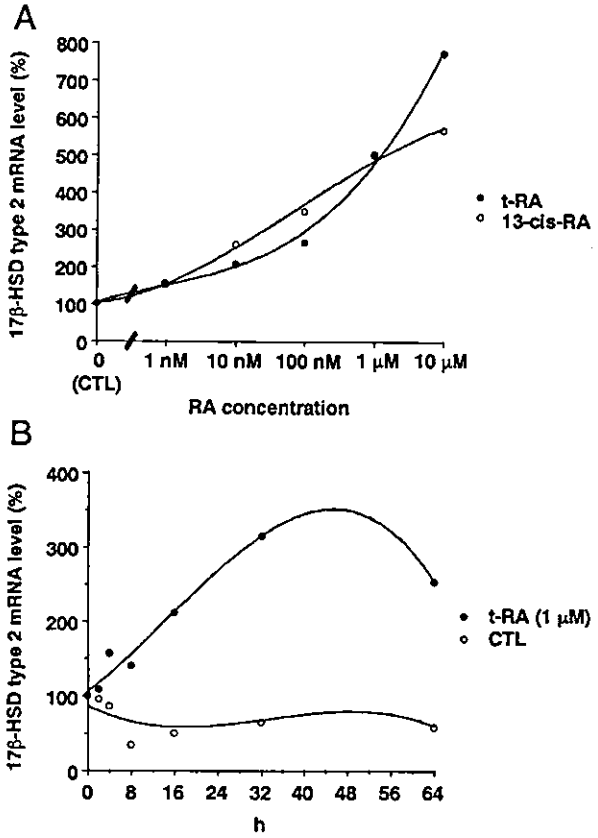

FIG. 6. A, Dose response of t-RA and 13-*cis*-RA on the levels of 17β-HSD type 2 mRNA in RL95–2 cells. The cells were treated with t-RA (closed bars) or 13-*cis*-RA (open bar) at six different concentrations (0, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM for 32 h. The levels of 17β-HSD type 2 mRNA were evaluated as percent change, compared with the control (nontreatment for 32 h). B, Time course of t-RA action on the level of 17β-HSD type 2 mRNA in RL95–2 cells. The cells were treated with (*closed circles*) or without (*open circles*) t-RA (1 μM) for 0, 2, 4, 8, 16, 32, and 64 h. The levels of 17β-HSD type 2 mRNA were evaluated as percent change, compared with that of 0 h treated cells.

We then examined the effects of retinoids on the expression of 17β-HSD type 2 mRNA in cell lines derived from human endometrial carcinoma based on these findings above. In our present study, 17β-HSD type 2 mRNA was detected in RL95–2 and HEC-1A cell lines, but its level was much greater in RL95–2. Variability in the expression of 17β-HSD type 2 by retinoic acid in endometrial carcinoma cells may reflect the characteristics and differentiation of endometrial epithelial cells from which the tumor arose. We therefore examined the various effects of retinoids on 17β-HSD type 2 mRNA expression using this cell line. Retinoic acid derivatives and selective RA receptor agonists increased the level of 17β-HSD type 2 mRNA in RL95–2 cells in both a time- and dose-dependent manner. However, the induction by LG69, a selective RXR agonist was approximately 0.38 times that of TTNPB, a RAR agonist, which is not necessarily consistent with the correlation between RXRγ and 17β-HSD type 2 in endometrial carcinoma cases. These findings suggest that retinoid actions through RXRγ alone may not be sufficient to induce 17β-HSD type2 mRNA. Retinoid actions through the RXRγ-RARα complex may be important for this induction, but further investigations are required to clarify these discrepancies.

No other correlations were detected between retinoid receptors and ER and aromatase. These results all suggest that retinoids influence *in situ* estrogen metabolism of both normal and neoplastic human endometrium on the expression of 17β-HSD type 2. A significant correlation was also detected between RXRγ and PR LI ($P = 0.003$) in endometrioid endometrial adenocarcinomas. In the human breast cancer cell line, T-47D, RA treatment induced a decrease in the cellular PR concentration by decreasing the amounts of its receptor mRNA and protein, suggesting that RA is capable of modulating sensitivity to progestins (32). Savouret *et al.* (33) demonstrated that transcriptional progesterone receptor gene expression was induced by estrogens and decreased by progestins and RA. In our study, a significant inverse correlation was also detected between RXRγ LI and patient age ($r = 0.449$, $P = 0.015$); RXRγ LI was markedly decreased in patients older than 50 yr old, which may also be due to the decrement of progesterone following menopause. Retinoids are considered to be effective as chemopreventive and chemotherapeutic agents in a variety of human epithelial and hematopoietic neoplasms (12, 34, 35). Kudelka *et al.* (35) reported the favorable results obtained with RA-based treatment of patients with cisplatin-resistant metastatic endometrial adenocarcinoma. These clinical effects of retinoids may be partly due to increased 17β-HSD type 2 mRNA expression in carcinoma cells, which results in the decreased *in situ* availability of biologically active estrogen, and the stimulation of PR expression. This may also reduce tumor cell proliferation through RXRγ, but it awaits further investigation such as possible inhibition of other subtypes of 17β-HSD including type 1 and type 3 and the correlation of the findings with percentage of body fat mass of individual patients for clarification.

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In vivo potentiation of human oestrogen receptor α by Cdk7-mediated phosphorylation

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Phosphorylation of the Ser[118] residue in the N-terminal A/B domain of the human oestrogen receptor α (hERα) by mitogen-activated protein kinase (MAPK), stimulated via growth factor signalling pathways, is known to potentiate ERα ligand-induced transactivation function. Besides MAPK, cyclin dependent kinase 7 (Cdk7) in the TFIIH complex has also been found to potentiate hERα transactivation *in vitro* through Ser[118] phosphorylation. To investigate an impact of Cdk7 on hERα transactivation *in vivo*, we assessed activity of hERα in a wild-type and *cdk7* inactive mutant *Drosophila* that ectopically expressed hERα in the eye disc. Ectopic expression of the wild-type or mutant receptors, together with a green fluorescent protein (GFP) reporter gene, allowed us to demonstrate that hERα expressed in the fly tissues was transcriptionally functional and adequately responded to hERα ligands in the patterns similar to those observed in mammalian cells. Replacement of Ser[118] with alanine in hERα (S118A mutant) significantly reduced the ligand-induced hERα transactivation function. Importantly, while in *cdk7* inactive mutant *Drosophila* the wild-type hERα exhibited reduced response to the ligand; levels of transactivation by the hERα S118A mutant were not affected in these inactive *cdk7* mutant flies. Furthermore, phosphorylation of hERα at Ser[118] has been observed *in vitro* by both human and *Drosophila* Cdk7. Our findings demonstrate that Cdk7 is involved in regulation of the ligand-induced transactivation function of hERα *in vivo* via Ser[118] phosphorylation.

Introduction

It is thought that most of the wide variety of oestrogen action is mediated through the transcriptional control of target genes by nuclear oestrogen receptor (ER) (Couse & Korach 1999; Ciana *et al.* 2003). The two subtypes of ER, α and β, belong to the nuclear receptor superfamily and act as ligand-induced transcription factors. As in other nuclear receptor superfamily members, structure of ER proteins is divided into five or six functional domains (designated as A to E/F domains). The highly conserved DNA binding domain is located in the C domain, while the ligand-binding domain (LBD) is mapped to the E/F domain. Transactivation function is present in the N-terminal A/B domain (AF-1) and in the C-terminal LBD (AF-2) (Kumar *et al.* 1987; Tora *et al.* 1989). Although both AF-1 and AF-2 are involved in the ligand-dependent transactivation function of ERs, AF-1 is constitutively active, while AF-2 activity is dependent on ligand binding (Endoh *et al.* 1999; Kobayashi *et al.* 2000; Watanabe *et al.* 2001). AF-1 and AF-2 domains have distinctive properties and their activities may depend on cell type and promoter context (Kumar *et al.* 1987; Tora *et al.* 1989).

ER target gene promoters contain oestrogen-response elements (EREs) that are recognized and directly bound by ER homo- or hetero-dimers followed by chromatin remodelling, presumably by recruited ATP-dependent chromatin remodelling complexes (Belandia & Parker 2003; Kitagawa *et al.* 2003). ERE-bound liganded ERs also induce recruitment of a number of histone acetyltransferase (HAT) and non-HAT cofactors that activate transcription (McKenna & O'Malley 2002). HAT coactivator complexes, CBP/p160 (Onate *et al.* 1995; Kamei *et al.* 1996; Chen *et al.* 1997; Spencer *et al.* 1997) and TRRAP/GCN5 (Yanagisawa *et al.* 2002), and non-HAT DRIP/TRAP complexes (Fondell *et al.* 1996;

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Yuan *et al.* 1998; Naar *et al.* 1999; Rachez *et al.* 1999) are thought to act as common coactivator complexes for ERs as well as for other DNA-binding transcription factors. Therefore, ligand binding leads to structural alteration and switch of ER function from transcriptional repression to transcriptional activation via the recruitment of coactivators (Shiau *et al.* 1998; Freedman 1999; Glass & Rosenfeld 2000; Metivier *et al.* 2003).

It is well known that phosphorylation of ERα modulates the activity of both AF-1 and AF-2 (Ali *et al.* 1993; Le *et al.* 1994; Kato *et al.* 1995; Chen *et al.* 2000). Among sites of potential phosphorylation, Ser^{118} residue (S118) in the hERα AF-1 domain has been particularly intensively studied with regard to the state of its phosphorylation and consequent potentiation of AF-1 activity. We have previously demonstrated that Ser^{118} is phosphorylated by ERK, a MAPK activated by the epidermal growth factor (EGF) or insulin-like growth factor (IGF) signalling, that results in the AF-1 potentiation in cultured cells (Kato *et al.* 1995). More recently, Chen and colleagues have shown that Cdk7 also phosphorylates hERα Ser^{118} in an oestrogen-dependent manner and enhances ERα transactivation in mammalian cells in culture (Chen *et al.* 2000). As Cdk7 is a key subunit of the basal transcription factor TFIIH complex (Frit *et al.* 1999; Egly 2001), it has been suggested that this phosphorylation takes place when TFIIH is recruited adjacent to hERα, presumably in the transcription initiation complex. Therefore, accumulating evidence suggests that phosphorylation of hERα Ser^{118} may play a significant role in regulation of AF-1 activity. However, the physiological role of Ser^{118} phosphorylation and associated kinases in hERα function remain to be established *in vivo*.

In *Drosophila melanogaster*, at least 20 members of the nuclear receptor (NR) family, such as the ecdysone receptor (EcR), have been genetically identified that, similar to the vertebrate NRs, are thought to transcriptionally control expression of target genes (Talbot *et al.* 1993; Baker *et al.* 2003). Recently, we reported that human androgen receptor ectopically expressed in *Drosophila* tissues was transcriptionally active and responsive to AR agonists and antagonists (Takeyama *et al.* 2002). In the present study, to assess an impact of Ser^{118} phosphorylation by Cdk7 and related kinases on hERα activity *in vivo*, we generated transgenic *Drosophila* lines in which hERα was ectopically expressed in specific *Drosophila* tissues using a GAL4/UAS system (Brand & Perrimon 1993). hERα expressed in the fly was transcriptionally functional and responded adequately to ER ligands, as expected from mammalian studies. Apparently, for its transactivation function in these transgenic flies, hERα recruited endogenous co-activators, such as those shown to be homologous to mammalian CBP and AIB1 (Akimaru *et al.* 1997; Bai *et al.* 2000). We found that replacement of S118 with alanine residue (S118A) in hERα resulted in the marked reduction of ligand-induced hERα transactivation in transgenic fly eye disc. Furthermore, in a *cdk7* inactive mutant *Drosophila* (*cdk7*^{a}) (Larochelle *et al.* 2001), transactivation by the wild-type but not the S118A hERα was significantly reduced. In addition, both human and *Drosophila* recombinant Cdk7 were equally able to phosphorylate hERα at Ser^{118} *in vitro*. We have also shown that Cdk7 acts as a co-activator of hERα transactivation in transfected cells in culture. Therefore, our results provide for the first time genetic evidence that phosphorylation of Ser^{118} potentiates transcriptional activity of hERα and that Cdk7 is involved in regulation of the ligand-induced transactivation function of hERα *in vivo* through Ser^{118} phosphorylation.

Results

hERα in *Drosophila* is transcriptionally functional

Our previous studies showed that human androgen receptor ectopically expressed in *Drosophila* tissues was adequately functional (Takeyama *et al.* 2002). We have utilized the same strategy to generate transgenic *Drosophila* expressing hERα together with ERE-dependent green fluorescent protein (GFP) as a reporter gene. Wild-type hERα (HEG0), AF-1 (HE15) or AF-2 (HE19) domains (as illustrated in Fig. 1A) were ectopically expressed in photoreceptor cells under control of the glass multimer reporter (*GMR*) gene promoter (Moses & Rubin 1991) using the *Drosophila melanogaster GAL4-UAS* system (Brand & Perrimon 1993). The eye disc, one of several larval discs in *Drosophila*, has been shown to be an effective model to assess Cdk7 function as a cell survival signal. Expression of hERα proteins was estimated by staining with immunofluorescent antibody. Levels of GFP reporter expression in respective eye discs were quantified by green fluorescence and normalized against the levels of ERα protein to determine fold of activation.

Dietary administration of 17β-oestradiol (E2) for 5 days from hatching remarkably induced GFP expression (Fig. 1B). The partial oestrogen agonist tamoxifen (TAM) and pure antagonist ICI182.780 exhibited partial oestrogenic and anti-oestrogenic actions, respectively, similar to that observed in mammals (McDonnell *et al.* 1995). E2-dependent (AF-2) and -independent (AF-1) transactivation functions were observed in the C-terminal-LBD and N-terminal A/B domain expressing transgenic flies, respectively, as expected from previous studies (Kumar *et al.* 1987; Tora *et al.* 1989; Kobayashi *et al.* 2000; Watanabe

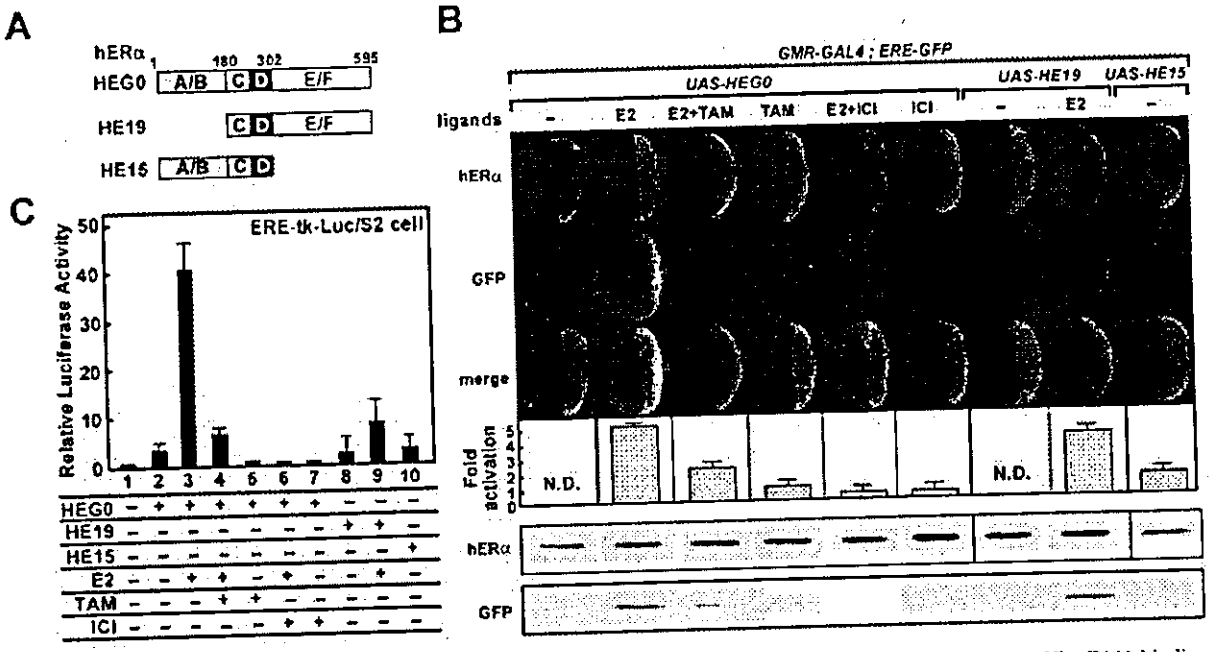

Figure 1 Ligand dependent transactivation of hERα in *Drosophila*. (A) Schematic representation of hERα constructs. The DNA binding domain (DBD) is located in the C domain (grey box). The transactivation function-1 (AF-1) region is located in the N-terminal A/B domain (blue box), while the transactivation function-2 (AF-2) region is located in the C-terminal E/F domain (white box) that also contains the ligand binding domain (LBD). (B) Ligand-dependent transactivation of hERα mutants in eye imaginal discs. Expression of hERα mutants in third instar larva eye discs driven by *GMR-GAL4* was detected with ERα antibodies (B10 or HC-20) (red). Transactivation of hERα mutants was estimated by GFP expression (green). The anterior is to the right. Bottom panels: hERα and GFP expression in four pairs of adult heads as detected by Western blotting. Fold-activation was calculated using hERα expression levels as normalizing factor. Ligands, 10^{-3} M 17β-oestradiol (E2), 10^{-2} M tamoxifen (TAM), and 10^{-2} M ICI 182.780 (ICI), were added in 100 μL of vehicle on top of 10 mL of the medium before hatching. Flies were kept at 25 °C. (C) Measurement of hERα mutants transactivation in Schneider cells. Schneider cells were transfected with hERα mutant expression plasmids, Actin-GAL4 plasmid, ERE-tk-luc reporter plasmid and pRL-CMV internal control plasmid in the presence or absence of 10^{-8} M E2, 10^{-8} M TAM or 10^{-8} M ICI. Firefly luciferase activity (ERE-tk-luc) was measured and normalized against Renilla activity (pRL-CMV-luc) as an internal control. Data are shown as the average and standard deviation of three independent experiments.

et al. 2001). Similar hER ligand effects and hERα AF-1 and AF-2 activities were observed in Schneider (S2) cells derived from *Drosophila* embryos (Fig. 1C). These data indicated that hERα ectopically expressed in *Drosophila* tissues was adequately functional in ligand-induced transactivation, presumably through recruitment of endogenous co-regulators. Therefore, it appears that human steroid receptors ectopically expressed in *Drosophila* retain their transactivation function.

Co-activation of hERα by *Drosophila* CBP and p160 HAT homologues

As hERα was transcriptionally functional in insect cells in culture and in *Drosophila* eye disc cells *in vivo*, ability of endogenous fly co-activators to modulate hERα transactivation was assessed in mutant flies deficient for *Drosophila* homologues of mammalian p160 (*tai*) or CBP (*nej*) (Akimaru *et al.* 1997; Bai *et al.* 2000). The oestrogen-induced transactivation function of hERα was clearly reduced in both of these mutants without affecting levels of hERα expression (Fig. 2). These data suggest that *Drosophila* homologue of the mammalian p160/CBP HAT complex acts as a co-activator of hERα in the fly cells. This was further confirmed by the observation of enhanced hERα transactivation in flies over-expressing TAI, *Drosophila* AIB1 homologue, in the eye disc.

The p160/CBP HAT complex has been shown to activate hERα AF-2 via the direct association of p160 family member proteins with helix 12 of the hERα LBD (Onate *et al.* 1995; Chen *et al.* 1997; Heery *et al.* 1997). However, little is known about the role of the

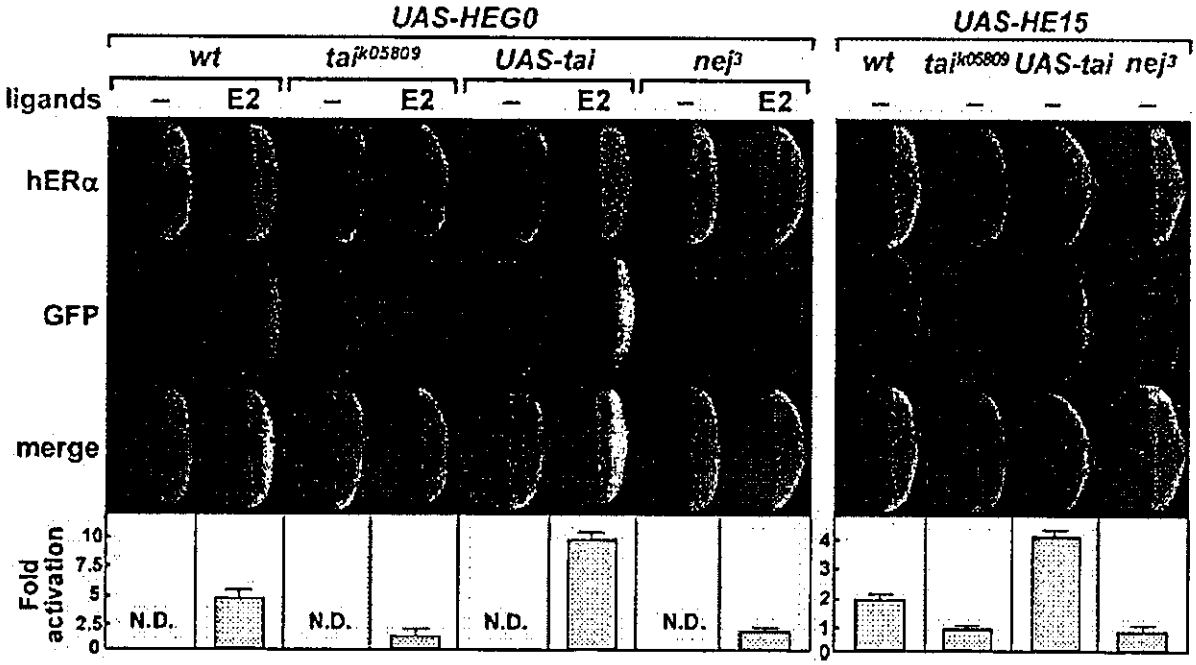

Figure 2 hERα transactivation regulated by *Drosophila* transcriptional co-activators. hERα expression (red) and transactivation (green) were visualized by immunostaining with ERα antibodies (B10 and HC-20) and GFP expression, respectively, in eye imaginal discs. Fly lines contained single copies of *GMR-GAL4*, *UAS-hERα* (*HEG0* or *HE15*) and *ERE-GFP* with or without heterozygous tai^{k05809}, *UAS-tai* or nej^3.

p160/CBP complex in modulation of hERα AF-1 activity. Although it is presumed that the complex bridges the AF-1 and AF-2 domains to synergistically enhance hERα transactivation function (Kobayashi *et al.* 2000), the p160/CBP complex was also able to enhance transcriptional activity of the AF-1 domain alone (i.e. the HE15 mutant). Indeed, similar patterns of AF-1 domain (HE15) and full-length hERα (HEG0) transactivation in mutant flies (Fig. 2) suggest that hERα AF-1 activity is modulated *in vivo* by the p160/CBP co-activator complex.

Significant role of Ser^{118} in hERα function *in vivo*

In mammalian cells, the potentiation of hERα AF-1 by phosphorylation of the Ser^{118} residue has been well documented (Kato *et al.* 1995; Chen *et al.* 2000). However, the impact of Ser^{118} phosphorylation in hERα transactivation function has not yet been verified *in vivo*. We tested the significance of hERα Ser^{118} in the insect S2 cells transfected with hERα mutants containing a serine to alanine replacement at position 118 (HE457, HE15/457) (Fig. 3A and 3B). These mutants exhibited decreased transactivation capacities even though levels of the mutant expression appeared to be similar to that of wild-type hERα.

We then examined the role of Ser^{118} in hERα function in transgenic flies (Fig. 3C). Although mutant and wild-type hERα expression levels in third instar larval eye discs were indistinguishable, a clear reduction in GFP induction was observed in the alanine replacement mutants. These findings provided evidence that the Ser^{118} residue played a pivotal role in hERα transactivation *in vivo*.

In vivo potentiation of hERα by Cdk7-mediated phosphorylation at Ser^{118}

As it is likely that the Ser^{118} residue could be phosphorylated by a number of endogenous protein kinases to support hERα transactivation, we studied the ability of dCdk7 to phosphorylate hERα at Ser^{118} *in vitro* and *in vivo*. The serine/threonine kinase Cdk7 is indispensable for transcription initiation by RNA polymerase II as an essential component of the transcription factor TFIIH complex (Frit *et al.* 1999; Egly 2001). $dcdk7^{ts}$ mutant flies express a temperature-sensitive Cdk7 mutant that is inactive at temperatures at or above 30 °C (Larochelle *et al.* 2001). We assessed transactivation function of HEG0 and HE457 in these $dcdk7^{ts}$ mutant flies (Fig. 4, left panel). Oestrogen-induced transactivation of

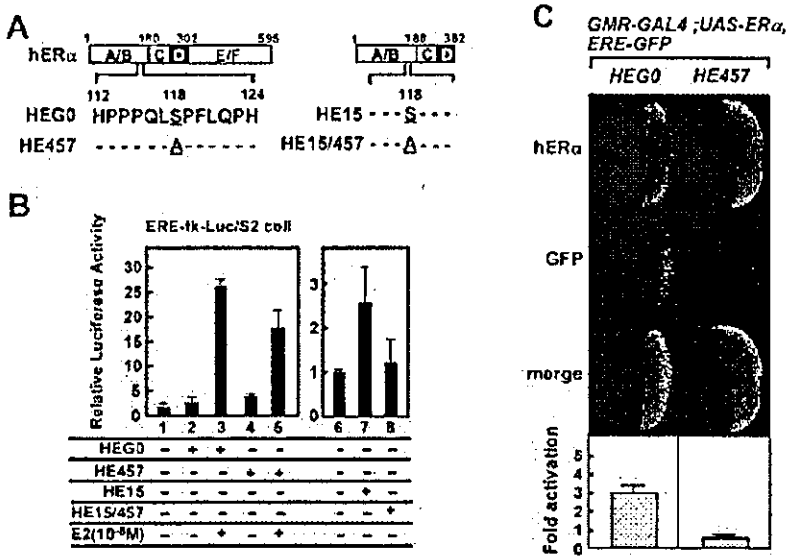

Figure 3 hERα transactivation is regulated by phosphorylation at Ser[118] in *Drosophila*. (A) Schematic representation of hERα mutant constructs. Ser[118] residue is the main phosphorylation site. (B) Transactivation of HEG0 and HE15 mutants in Schneider cells. Schneider cells were transfected with ERE-tk-luc reporter plasmid, Actin-GAL4 plasmid and each hERα mutants, and then incubated with or without 10^{-8} M E2. Luciferase activity data are shown as the average and standard deviation of three independent experiments. (C) Expression (red) and transactivation (green) of hERα mutants in eye imaginal discs. Fold-activation is represented as described (Fig. 1 legend). Genotypes are *GMR-GAL4/SM. UAS-hERα, ERE-GFP/TM3.*

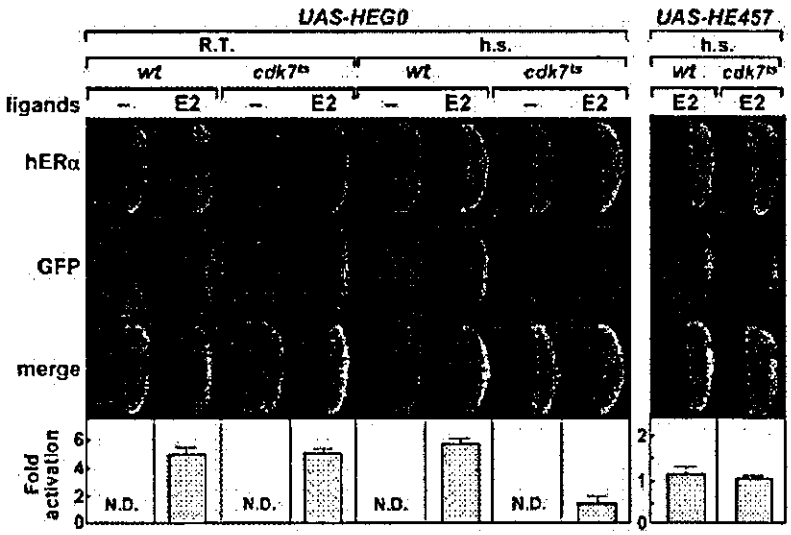

Figure 4 hERα transactivation is enhanced by *Drosophila* Cdk7 through phosphorylation of Ser[118]. hERα expression (red) and transactivation (green) in eye imaginal discs containing single copies of *GMR-GAL4, ERE-GFP* and *UAS-hERα* (*HEG0, HE457*) with or without heterozygous *cdk7*$^{+}$. *cdk7*^{ts}, the temperature-sensitive *cdk7*^{P140S} gene, was introduced into the *Df(1)JB254-Pw*$^{-}$*[snf*$^{+}$, *dhd*$^{+}$] (*cdk7*deficient) background. Flies were then incubated at 25 °C (room temperature) or 30 °C (h.s.) for 24 h in medium containing E2. GFP expression levels are represented as described.

HEG0 in *dcdk7*^{ts} flies was significantly reduced at 30 °C in comparison with that at room temperature (25 °C). In contrast, HE457 transactivation in *dcdk7*^{ts} flies was not affected by exposure to high temperatures (Fig. 4, right panel). These results indicate that Cdk7 potentiated hERα transactivation *in vivo* through Ser[118] phosphorylation.

To further confirm this conclusion, we examined whether hERα Ser[118] is a substrate for dCdk7 *in vitro*. A recombinant GST-fused hERα segment (amino acids 56–180) chimera protein expressed in *E. coli*, and dCdk7 and hCdk7 expressed in 293T cells were used for the *in vitro* phosphorylation assay (Fig. 5A). GST-fused human retinoic acid receptor α1 (hRARα1), a well-characterized substrate for the mammalian Cdk7 (Rochette-Egly *et al.* 1997) was used as a positive control. dCdk7 and hCdk7 were equally capable of phosphorylating hERα and hRARα1. However, the Cdk7 phosphorylation was clearly reduced when the S118A mutant (HE457) was used as a substrate (Fig. 5B).

Activation of the hERα S118A mutant by *Drosophila* AIB1 homologue

Finally, using a fly line with ectopical over-expression of *Drosophila* AIB1 homologue (TAI) in the eye disc, we

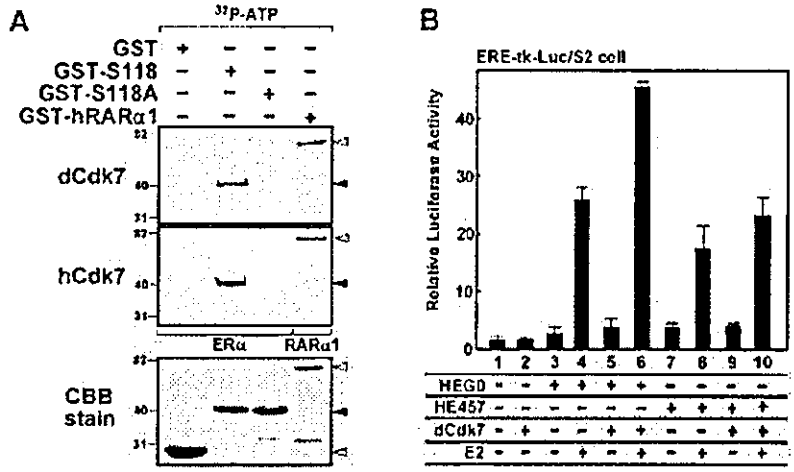

Figure 5 *In vitro* phosphorylation of hERα at Ser[118] by dCdk7. (A) Ten micrograms of GST-fused hERα (amino acids 56–180) and GST-fused hRARα1 were incubated with 9 μg dCdk7 or hCdk7. Phosphorylation and expression of GST-fused hERα amino acids (open arrow head), GST-fused hRARα1 (black arrow head), and GST (grey arrow head) were detected by autoradiography and CBB staining, respectively. (B) Schneider cells were co-transfected with 0.5 μg dCdk7 expression plasmid, 0.5 μg ERE-tk-luc reporter plasmid, 0.2 μg actin-GAL4 plasmid and hERα mutants and then incubated with or without 10^{-8} M E2. Luciferase activity data are shown as the average and standard deviation of three independent experiments.

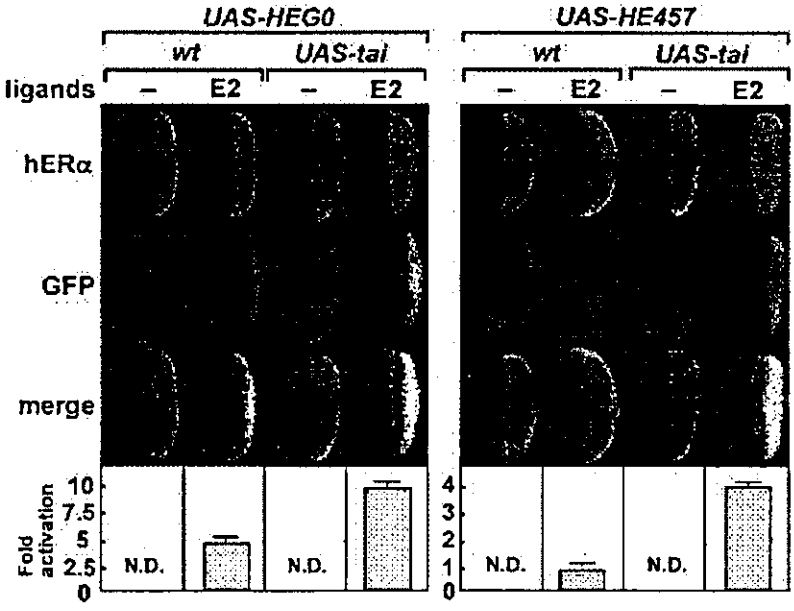

Figure 6 TAI enhancement of hERα transactivation is not dependent on Ser[118] phosphorylation status. hERα expression (red) and transactivation (green) in eye imaginal discs of either HEG0 or HE457 expression lines are shown. TAI is also expressed driven by *GMR-GAL4*.

addressed a question whether enhancement of hERα transactivation by the p160/CBP complex is dependent on the receptor Ser[118] phosphorylation status. Although the hERα S118A mutant was less effective in the ligand-induced transactivation, TAI significantly enhanced transcriptional activity of both the mutant and the wild-type receptor (Fig. 6). This suggests that modulation of the ligand-induced hERα transactivation by the p160/CBP co-activator complex does not depend on the receptor phosphorylation.

Discussion

hERα expressed in *Drosophila* is functional in ligand-induced transactivation

We have previously shown that the human androgen receptor (hAR) ectopically expressed in *Drosophila* tissues was transcriptionally functional and responsive to hAR ligands similar to that in mammalian cultured cells and intact tissues (Takeyama *et al.* 2002). In the present study, we utilized the same approach and demonstrated