EXPRESSION OF SEC61α IN F9 AND P19 TERATOCARCINOMA CELLS AFTER RETINOIC ACID TREATMENT

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ABSTRACT

Nascent procollagen peptides and other secretory proteins are transported across the endoplasmic reticulum (RE) membrane through a protein-conducting channel called the translocon. Sec61α, a multispanning membrane translocon protein, has been implicated as essential for translocation of polypeptides chains into the cisterns of the ER. However, it is not known whether Sec61α is ubiquitously expressed in collagen producing teratocarcinoma cells. Furthermore, the production, expression, and utilization of Sec61α may depend on the cell differentiation stage. Stem cells from many cultured teratocarcinoma cell lines such as F9 and P19 cells are capable of differentiation in response to low retinoic acid concentrations. This differentiation of the tumorigenic stem cells results in tumorigenicity loss. For this study, mouse F9 and P19 teratocarcinoma cells were grown in culture medium treated with or without retinoic acid. Expression of Sec61α was determined by reverse trancriptase polimerase chain reaction (RT-PCR). In untreated conditions, F9 cells expressed undetected Sec61α amounts. It was also demonstrated that Sec61α expression is stimulated in F9 cells after retinoic acid treatment for 72 hours. No changes were found in Sec61α expression in P19 cells after retinoic acid treatment. These data indicate that the expression of Sec61α is enhanced with retinoic acid induced differentiation of F9 teratocarcinoma cells.

Key words: Sec61α, expression, F9 teratocarcinoma cells, retinoic acid.

RESUMO

Expressão gênica de Sec61α após tratamento com ácido retinóico em células de teratocarcinoma F9 e P19

Peptídeos nascentes de pró-colágeno e outras proteínas são transportadas por intermédio da membrana do retículo endoplasmático por um canal de transporte de proteínas chamado de *tanslocon*. Sec61α, uma proteína transmembrânica do *translocon*, tem sido apontada como essencial para translocação de cadeias nascentes de polipeptídeos para as cisternas do retículo endoplasmático. Entretanto, não se sabe se Sec61α é constitutivamente expressa em células de teratocarcinoma produtoras de colágeno. Além disso, a expressão e a utilização de Sec61α podem ser dependentes do estágio de diferenciação celular. Células progenitoras pluripotentes obtidas de muitas linhagens de células de teratocarcinoma, incluindo, por exemplo, células

F9 e P19, são capazes de diferenciação em resposta a baixas concentrações de ácido retinóico. Essa diferenciação de células tumorais causa perda de sua carcinogenicidade. Para este estudo, células de teratocarcinoma F9 e P19 de camundongos foram cultivadas em meios de cultura tratados com ou sem ácido retinóico. A expressão de Sec61α foi determinada pelo método de "reverse transcriptase-polymerase chain reaction" (RT-PCR). Em condições não tratadas, células F9 expressaram quantidades não detectáveis de Sec61α. De forma similar, experimentos de RT-PCR demonstraram que a expressão gênica de Sec61α é estimulada nas células F9 após tratamento com ácido retinóico por 72 horas. Não foram detectadas diferenças na expressão de Sec61α nas células P19 após tratamento com ácido retinóico. Esses dados demonstram que a expressão de Sec61α é aumentada em células de teratocarcinoma F9 após diferenciação com ácido retinóico.

Palavras-chave: Sec61α, expressão gênica, células de teratocarcinoma F9, ácido retinóico.

INTRODUCTION

Retinoic acid (RA) is a natural acidic derivative of vitamin A (retinol), which can affect proliferation and differentiation of a variety of cell types, and pattern formation during development (Strickland & Mahdavi, 1978; DeLuca, 1992). Retinoic acid can mimic the action of the polarizing zone activity in the developing chick limb (Tickle *et al.*, 1982, 1985), and cause specific alterations in the proximal-distal pattern of regenerating amphibian limbs (Tickle *et al.*, 1985). In addition, retinoic acid can suppress the process of carcinogenesis *in vivo* (Moon & Metha 1990; Shklar *et al.*, 1980) and inhibit the development of the transformed phenotype *in vitro* (LaRosa & Gudas, 1988).

Studies in experimental animals have demonstrated that retinoids suppress carcinogenesis in a variety of epithelial tissues, including skin, trachea, lung, and oral mucosa (Moon & Metha, 1990; Shklar *et al.*, 1980). Clinical studies have demonstrated the efficacy of retinoids in suppressing oral premalignant lesions, second primary carcinomas in patients with head and neck or lung cancer, and also skin cancers in patients with xeroderma pigmentosum (Hong *et al.,* 1986; Kraemer *et al.*, 1988; Smith *et al.*, 1992; Kikugawa *et al.*, 2000; Wan *et al.*, 2001). In tissue culture systems, RA induces differentiation of murine F9 teratocarcinoma cells (Boylan & Gudas, 1991; Fischer *et al.*, 2000; Wakabayashi *et al.*, 2000; Kubota *et al*., 2001) and can also influence differentiation of HL-60 human promyelocytic leukemia cells (Brietman *et al*., 1980; Treigyte *et al*., 2000; Hussain *et al*., 2000), melanoma cells (Lotan & Lotan, 1980) and human neuroblastoma cells (Haussler *et al*., 1983).

The F9 cells comprise a cultured cell line derived from a mouse testicular teratocarcinoma (Strickland & Mahdavi, 1978). The F9 murine teratocarcinoma stem cells resemble the pluripotent inner cell mass cells of mouse blastocysts. Thus, F9 cells provide an important model system with which to study critical early events in mouse development (Strickland & Mahdavi, 1978; Fischer *et al*., 2000; Kubota *et al*., 2001). These embryonal carcinoma cells, which undergo very limited differentiation under normal culture conditions, can be induced to differentiate into parietal endodermal cells (primitive endoderm-like cells) in the presence of retinoic acid and dibutyryl cAMP (Strickland *et al*., 1980; Kubota *et al*., 2001). The P19 cells are a euploid (40:XY) teratocarcinoma cell line derived from an embryonal carcinoma induced in a C3H/He strain mouse (Wakabayashi *et al*., 2000). These pluripotent cells, which differentiate poorly under normal culture conditions, can be induced to differentiate into neuronal, glial, or fibroblast-like cells in the presence of retinoic acid (Wakabayashi *et al*., 2000; Fernandez-Rachubinski *et al*., 2001).

Secretory proteins produced by teratocarcinoma cells are transported across the endoplasmic reticulum (ER) membrane through a protein-conducting channel. This channel is comprised of ER associated proteins called translocon, which includes the Sec61α complex (Simon & Blobel, 1991; Kellaris *et al*., 1991; Rapoport *et al*., 1999). Sec61α is a multispanning membrane protein and the largest component of the Sec61p complex. This protein is considered essential for translocation of polypeptide chains through this channel into the ER cisterns of the ER (Gorlich *et al*., 1992; Greenfield & High, 1999). Sec61α expression may depend on the cell differentiation stage. However, it is not known whether Sec61α is ubiquitously expressed in collagen-producing cells, and under stress conditions such as heat shock.

Stem cells from many cultured teratocarcinoma cell lines, such as mouse F9 and P19 cells, are capable of differentiation in response to low retinoic acid concentrations (Wang & Gudas, 1990; Boylan & Gudas, 1991). This tumorigenic stem cell differentiation results in the loss of tumorigenicity (Jetten *et al*., 1992; Lotan, 1993). Therefore, teratocarcinoma cell lines provide a model with which to examine the association between the process of cell differentiation and Sec61α expression. This study aimed to investigate the expression of Sec61α in F9 and P19 cells following retinoic acid and heat shock treatment.

MATERIAL AND METHODS

F9 teratocarcinoma cells

The F9 teratocarcinoma cells were obtained from the American Type Tissue Collection and grown to confluence in 75 cm^2 plastic flasks using Dulbecco's modified Eagle's medium, 10% FBS, 1.16 g/liter glutamine, and 100 mg/ml streptomycin at 37°C in 5% CO_2. The F9 stem cells (1 x 10^5 cells) were plated on gelatinized tissue culture plates containing 10 ml of medium, and induced to differentiate into parietal endoderm cells after treatment with all-*trans* retinoic acid (10^{-6} M) for 4 days. Retinoic acid was added 24 h after plating. All trans RA (Sigma, St. Louis, MO, USA) stock solution was dissolved in 100% ethanol and stored in the dark. The controls were treated with vehicle alone (ethanol) and processed in parallel with the experimental groups. The cells were grown under normal conditions (37°C) or at 43°C (heat shock) for 90 min.

P19 teratocarcinoma cells

Mouse P19 teratocarcinoma cell lines were obtained from the American Type Tissue Collection and grown to confluence in 75 cm^2 plastic flasks using Alpha modified Eagle's medium, 10% FBS, 1.16 g/liter glutamine, and 100 mg/ml streptomycin at 37°C in 5% CO_2. These P19 cells were induced to differentiate into fibroblast-like cells after treatment with all-*trans* retinoic acid (10^{-6} M) for 4 days as described above for F9 cells.

MDCK cells

The MDCK (Madin-Darby canine kidney) cell line was derived from a kidney of an apparently normal adult female cocker spaniel. The line was established in a medium consisting of 0.5% lactalbumin hydrolysate in 95% Earle's BSS (balanced salt solution) and 5% bovine serum. The initial cells appeared to be fibroblast-like, and were removed from the culture six times at approximately 7 day intervals. MDCK cell lines were obtained from the American Type Tissue Collection and grown to confluence in 75 cm^2 plastic flasks using Eagle's modified essential medium with Earle's BSS, 10% FBS, 1.16 g/liter glutamine, and 100 mg/ml streptomycin at 37°C. The MDCK cells were grown to confluence, frozen, and recovered for the experiments. Cells between the third and fourth passage were utilized for the experiments. These cells were used in this study as the control.

RNA extraction

The RNA was extracted from F9, P19, and MDCK cells using standard procedures (Wang & Gudas, 1990). The cells were collected by centrifugation at 300 x g for 5 min at 4°C and washed in ice-cold PBS. Fifteen ml of prechilled denaturing solution (40% guanidine thiocyanate in CSB buffer, Promega, Madison, WI) was then added to the cells. The samples were then disrupted with a high speed homogenizer (Brinkmann Polytron, New Haven, CT) for 30 seconds. After homogenization, 1.2 ml of 2M sodium acetate, pH 4.0, was added to the samples. The samples were extracted with 12 ml of 1:1:1 phenol/chloroform/isoamyl alcohol mixture (Sigma Co.). The samples were transferred to a 50 ml thick-walled polypropylene tube (DEPC, diethyl-pyrocarbonate-treated) and centrifuged at 10,000 x g for 20 min at 4°C. The top aqueous phase which contains the RNA was removed and transferred to a fresh DEPC-treated tube. The RNA was precipitated from the aqueous phase by adding an equal amount of isopropanol to each sample and incubating overnight at –20°C. The RNA pellet was collected by centrifugation at 10,000 x g for 15 min at 4°C, resuspended in RNase-free water, and stored at –20°C. The RNA was resuspended in nuclease-free water, and RNA concentrations were determined by spectrophotometry at 260 nm. The RNA integrity was verified by visualization of sized-resolved RNA on ethidium bromide stained 1% agarose gels. The RNA yields ranged from 0.2 to 71 µg/µl.

PCR amplifications

One to 5 µg of extracted RNA was resuspended in 13 µl of DEPC-treated water. One µl of Oligo-dT (10 µM) was added to each sample. The samples were immediately heated to 70°C for 10 min and quenched on ice for 1 min. The following solutions

were immediately added to the samples: 2 µl of 10X synthesis buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, 20 mM $MgCl_2$), 1 µl of 10 mM dNTP mix, 2 µl of 0.1 M of DTT, and 1 µl of 200 U/ µl SuperScript II RT (BRL Inc.). The samples were incubated for 10 min at room temperature, transferred to 42°C, and incubated for an additional 50 min. Reverse transcription was terminated by placing the samples at 70°C for 15 min. To each sample was added RNaseH (1 µl), and the samples were incubated for 20 min at 37°C. The following solutions were then added to each sample: 8 µl of 10X synthesis buffer, 1 µl of upstream primer (10 µM), and 1 ml of downstream primer (10 µM). Primers were designed for homologous regions of rat and canine Sec61α (Gorlich *et al*., 1992). Upstream primer sequence was 5'-GAACTTCTG CAAAAAGGGTACGG-3' (23mer); the downstream primer sequence was 5'-TTCTACACTTCCAACAT CCCC-3' (21mer). The expected size of the amplified product was 410 base pairs (bp) (Gorlich *et al*., 1992). Following the addition of the primers, a wax pellet was added to each tube, and the tubes were incubated at 80°C for 7 min. The wax was allowed to harden, and 1 µl of Taq DNA polymerase (Promega Corp., Madison, Wisc.) and 68 µl of sterile distilled water were added on to the top of the wax. Thermal cycling consisted of one cycle of 6 min at 94°C, 2 min at 59°C, and 1 minute at 72°C, followed by 35 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C. The PCR products were electrophoresed on 7% polyacrylamide gels, and products were visualized by ethidium bromide staining and ultraviolet (UV) transillumination of the gel.

RESULTS

Under normal conditions, the F9 mouse teratocarcinoma stem cell line proliferates spontaneously and normally displays dense compact morphology of cells which tend to aggregate into colonies (Fig. 1A). In response to RA treatment, F9 differentiated from a homogeneous population of primitive endoderm cells into a population of more differentiated parietal endoderm cells (Fig. 1B), showing morphologically a more dispersed configuration and increased number of cellular processes.

In the absence of any treatment, the plated aggregates of P19 cells contained undifferentiated embryonal carcinoma cells that adhered to each other, forming small aggregates. Treatment of P19 cells with all-trans retinoic acid (10^{-6} M) for 4 days resulted in differentiation into fibroblast-like cells (results not shown). Treatment of MDCK cells with RA did not alter the fibroblast-like appearance of these cells (Fig. 2A and B).

The RNA extraction and RT-PCR amplification were performed in order to verify the expression of Sec61α after retinoic acid treatment. Since the primer utilized in this experiment is based on canine Sec61α cDNA sequence, MDCK cells were used in these experiments as control cells. The cDNA samples from MDCK and F9 cells amplified with both Sec61α (410 bp) and β-actin primers are shown in Fig. 3.

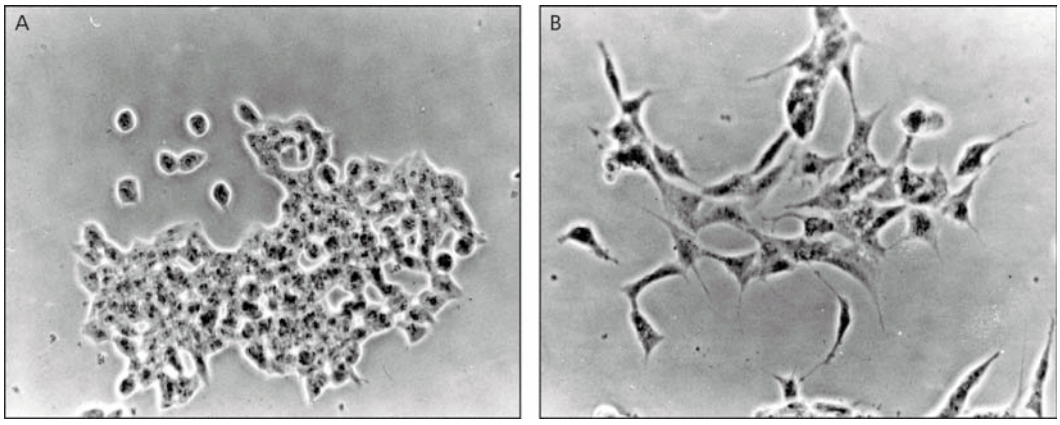

Fig. 1 — (A, B). Phase contrast photomicrographs of F9 teratocarcinoma cells illustrating the variation in morphology after treatment with retinoic acid. Cell aggregates were plated and grown in: A, normal medium (a-medium plus 10% FBS); and B, in medium containing 10^{-6} M retinoic acid (final magnification x 220).

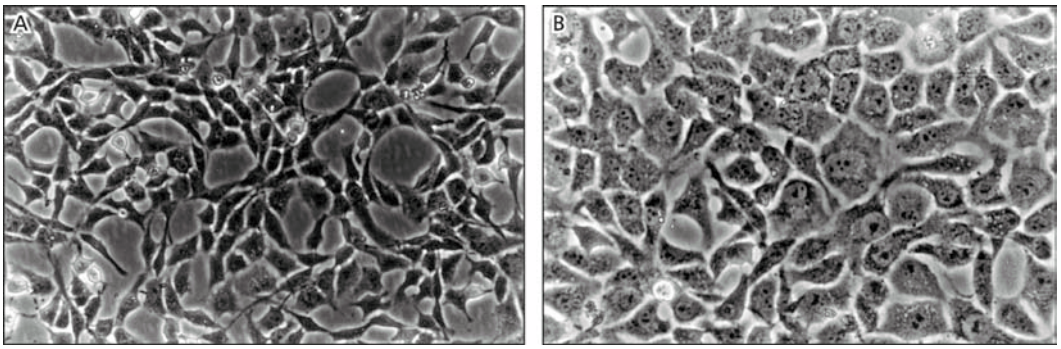

Fig. 2 — (A, B). Monolayer culture of MDCK (Madin-Darby canine kidney) cells showing abundant cytoplasm and a cuboidal appearance. Cell aggregates were plated and grown in: A, normal medium (a-medium plus 10% FBS) (final magnification x 220); and B, in medium containing 10^{-6} M retinoic acid (final magnification x 280).

An amplified product for Sec61α primer was detected in samples from both control and retinoic acid treated in MDCK cells (Fig. 3, lanes 1 & 2, respectively). In F9 cells, however, a band of 410bp (Sec61α primer) was detected only in samples from retinoic acid treated cells (Fig. 3, lane 4). The next experiment was performed to verify whether Sec61α expression is altered under different metabolic conditions such as heat-shock treatment (Fig. 4). Following treatment with retinoic acid, an amplified product of appropriate size for Sec61α primer was present in samples from both control (incubated at 37°C) and heat shocked (incubated at 43°C) cells (Fig. 4, lanes 3 & 4, respectively). The amplified product for Sec61α primer was not detected in samples from cells that were not treated with retinoic acid following heat-shock (Fig. 4, lane 2).

Finally, we investigated whether Sec61α expression was also altered following retinoic acid treatment in another teratocarcinoma cell line: P19 cells (Fig. 5). The amplified product for Sec61α primer from P19 cells was detected in both control and retinoic acid-treated samples (Fig. 5, lanes 1 & 2).

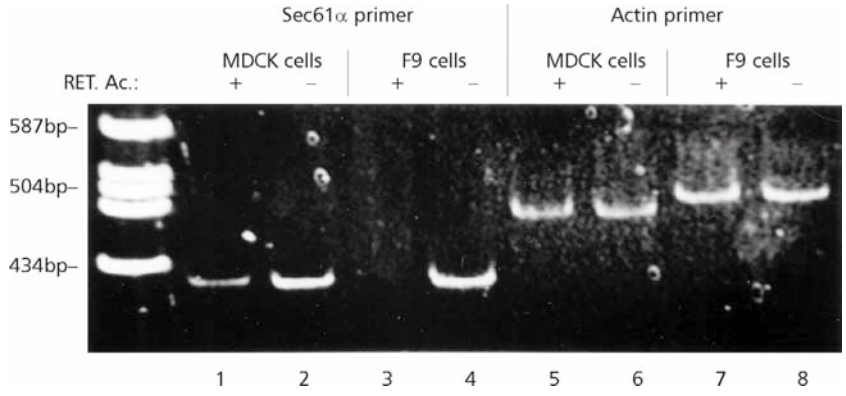

Fig. 3 — Expression of Sec61α in canine MDCK and mouse F9 cells following retinoic acid treatment. The PCR products from F9 cells untreated (lanes 3 & 7) and treated (lanes 4 & 8) with retinoic acid, and from MDCK cells also untreated (lanes 1 & 5) and treated (lanes 2 & 6) were electrophoresed on 7% polyacrylamide gels. The products amplified by the Sec61α primers (lanes 1-4) and control actin primers (5-8) were run side-by-side on gels, stained with ethidium bromide, and photographed. The expected size of the amplified product of Sec61α primer was 410 base pairs on the basis of gel electrophoresis. The expected size of the actin control was 490 base pairs.

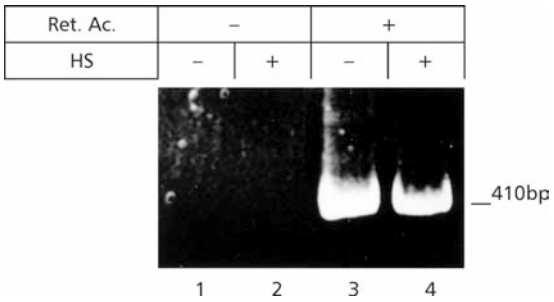

Fig. 4 — Expression of Sec61α in F9 cells following retinoic acid treatment and heat shock. Prior to RNA extraction, F9 cells were incubated at 37°C with (lanes 3 & 4) or without (lanes 1 & 2) retinoic acid for 4 days. The cells were then incubated for additional 90 min at 37°C (control cells, Lanes 1 & 3) or at 43°C (heat-shocked cells, lanes 2 & 4). The PCR products from F9 cells were electrophoresed on 7% polyacrylamide. The products amplified by the Sec61α primers treated with retinoic acid and heat-shock were run side-by-side on gels, stained with ethidium bromide, and photographed. The expected size of the amplified product was 410 base pairs on the basis of gel electrophoresis.

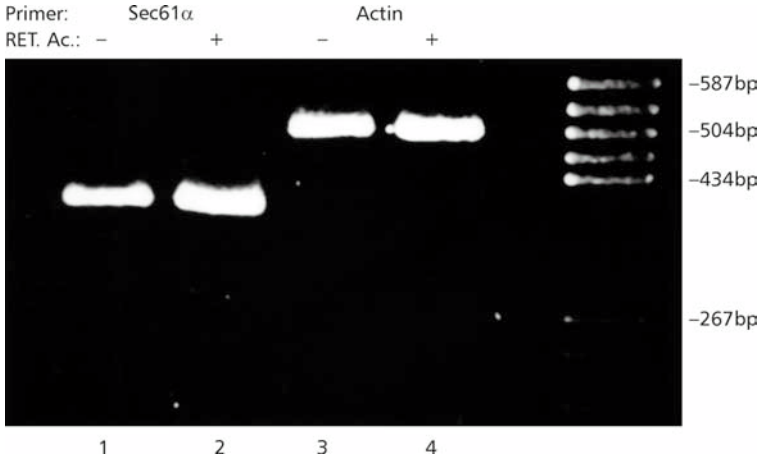

Fig. 5 — Expression of Sec61α in P19 cells following retinoic acid treatment. Prior to RNA extraction, P19 cells were incubated at 37°C with (lanes 2 & 4) or without (lanes 1 & 3) retinoic acid for 4 days. RT-PCR amplification was performed as previously described. The products amplified by the Sec61α primers (lanes 1 & 2) and control actin primers (3 & 4) were run side-by-side on the gels, stained with ethidium bromide, and photographed. The expected size of the amplified product of Sec61α was 410 base pairs on the basis of gel electrophoresis. The expected size of the actin control was 490 base pairs.

DISCUSSION

Retinoic acid is an established regulator of epithelial cell differentiation *in vivo* and *in vitro* (DeLuca, 1992; Zou *et al*., 1994). One of the major physiological functions of retinoic acid is to prevent squamous differentiation (Jetten *et al*., 1992; Lotan, 1993; Kubota *et al*., 2001; Fischer *et al*., 2000). Squamous metaplasia, which develops during vitamin A deficiency, can be reversed by supplementation with retinoic acid (Lotan, 1993). Furthermore, retinoic acid suppresses squamous cell differentiation that either occurs spontaneously or is induced by calcium ions in cultured normal keratinocytes or tracheal cells (Jetten *et al*., 1992; Kubota *et al*., 2001). In the presence of retinoic acid and dibutyryl cAMP, F9 cells can be induced to differentiate into parietal endodermal cells (primitive endoderm-like cells) (Strickland *et al*., 1980; Kubota *et al*., 2001).

The phenotype of the new cell type is characterized by synthesis of plasminogen activator,

laminin, and type IV collagen, and by low levels of alkaline phosphatase and lactase dehydrogenase. The cell type generated by this induction does not depend upon the continued presence of either compound, but the cAMP agents are active only on F9 cells treated with retinoic acid (Strickland & Mahdavi, 1978; Jetten *et al*., 1979). This differentiation of the tumorigenic F9 stem cells results in tumorigenicity loss (Strickland *et al*., 1980; Kubota *et al*., 2001). This differentiation response of F9 cells is irreversible, fairly synchronous, and rapid (within 24 to 48 hours) (Jetten *et al*., 1979; Kubota *et al*., 2001).

The molecular mechanisms by which retinoids exert such potent effects on the growth, differentiation, and neoplastic transformation of a variety of different cell types have not been elucidated, even though many of these retinoid effects have been known for over 60 years. There is some evidence that retinoids may act in a manner similar to that of steroid hormones (Lotan, 1980; Wan *et al*., 2001). Although the exact mechanism whereby RA regulates gene expression and differentiation is not completely understood, it is likely that high-affinity RA receptors and/or binding proteins are involved (Sato *et al*., 2001).

Previous study has investigated the production of Sec61α and other translocon components following retinoic acid treatment in F9 teratocarcinoma cells (Ferreira *et al*., 2002). The production of Sec61α was shown to markedly increase following retinoic acid treatment in treated F9 cells, whereas undetected Sec61α amounts were observed in untreated F9 cells. However, another translocon component, TRAM, was present in both retinoic acid-treated and control samples (Ferreira *et al*., 2002).

The relevance of undetectable levels of Sec61α in undifferentiated F9 cells is not known. However, Sec61α was essential for secretory protein translocation (Kellaris *et al*., 1991). The mechanism whereby these tumor cells secrete proteins without detectable Sec61α amounts is still not well understood. Among mammalian species, the cDNA sequence for the Sec61α gene is known for rats, dogs, and humans. However, there has also been no convincing evidence in any system that retinoic acid regulates Sec61α transcription.

In this study, the cDNA samples from F9 cells amplified with Sec61α primer were demonstrated in samples of cells treated with retinoic acid, but not in control cell samples. The PCR samples from P19 cells demonstrated an amplified product for Sec61α primer in retinoic acid treated and untreated samples. These findings may suggest that Sec61α expression depends on F9 teratocarcinoma cell differentiation into parietal endoderm cells following retinoic acid treatment. Whether the cell differentiation stage regulates Sec61α expression is still not clear.

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