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THIOREDOXIN GENE EXPRESSION IS TRANSCRIPTIONALLY UP-REGULATED BY RETINOL IN MONKEY CONDUCTING AIRWAY EPITHELIAL CELLS+

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SUMMARY: Using the differential hybridization technique, a cDNA clone, MT78, was isolated from the cDNA library of retinol-treated monkey tracheobronchial (TBE) epithelial cells. MT78 has a high sequence homology to human thioredoxin. The cDNA insert contains 506 nucleotides which encodes a peptide of 105 amino acids. The deduced peptide contains the highly conserved sequence Cys-Gly-Pro-Cys, found at the active site of all thioredoxins. The expression of the thioredoxin gene is stimulated 8-10 fold by vitamin A (retinol) in monkey TBE cells. The expression is significantly enhanced within 4 h after the vitamin A treatment and concurrent protein synthesis is not required for this enhancement. These results, in conjunction with the nuclear run-on transcriptional assay, support the conclusion that thioredoxin gene is transcriptionally up-regulated by retinol and/or its metabolites.

The major role of vitamin A (retinol) and its metabolites (retinoids) in the maintenance of the homeostasis of the conducting airway epithelium has been well recognized (1, 2). In a vitamin A deficient environment, the normal morphology of airway epithelium is altered; the cells take on a squamous appearance. This phenomenon, namely squamous cell metaplasia, is similar to the early lesions involved in the development of bronchogenic cancer (3, 4). It is not surprising that the prevention of such lesions with a vitamin A treatment would hinder epithelial carcinogenesis (3). Despite the importance of vitamin A's effect on epithelial cells, the underlying mechanisms are still poorly understood.

We are interested in the role of vitamin A in regulating airway epithelial cell functions. A serum-free hormone-supplemented medium has been developed to cultivate airway epithelial cells over a long period in vitro (5). In this serum-free culture condition, vitamin A is required for airway epithelial cells to express at least the mucous cell function. In the absence of vitamin A, airway epithelial cells express such squamous properties as cornification and keratinization. This process is reversible with the addition of vitamin A to the culture system. Therefore, this serum-

+ Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. M84643.

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Abbreviation used: TBE, tracheobronchial epithelial.

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free culture model offers an unique system to analyze systematically the effects of vitamin A on epithelial cell differentiation. In this communication, we used the culture system and the differential hybridization technique to isolate vitamin A-responsive genes. The cDNA clone MT78, which was isolated from the cDNA library of retinol-treated monkey tracheobronchial epithelial (TBE) cells, shares high homology with the human thioredoxin cDNA sequence.

Thioredoxin is found in organisms ranging from bacteria to human and is implicated in a wide variety of biochemical pathways, especially in signal transduction (6-8). The discovery that thioredoxin is one of the vitamin A-responsive genes may have far-reaching implications for the action of vitamin A in the regulation of airway epithelial cell differentiation. We describe here the isolation of the cDNA clone MT78 and the up-regulation of the gene transcription by retinol.

MATERIALS AND METHODS

Cell isolation and Culture Conditions. Rhesus monkey TBE cells were isolated and cultured in a serum-free, hormone-supplemented medium as described before (5, 9). The medium consisted of the F12 nutrients (GIBCO, Grand Island, NY) supplemented with insulin (5 μg/ml, Sigma, St. Louis, MO), transferrin (5 μg/ml, Sigma), epidermal growth factor (20 ng/ml, Upstate, Lake placid, NY), cholera toxin (20 ng/mlLists, Campbell, CA), dexamethasone (0.1 μM, Sigma), bovine hypothalamus extract (30 μg/ml, (9)), and with or without retinol (1 μM, Sigma). Primary cultures were maintained in this serum-free medium. Retinol was added at day 7.

mRNA Isolation, cDNA Library Construction and Screening. RNA was isolated by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski, et al. (10). Poly(A^+) mRNA isolation, cDNA library construction, and the differential (+/–) screening of the libraries were performed as described previously (11, 12).

DNA Sequence Analysis. Sequencing of the cloned cDNA was performed according to the dideoxynucleotide-chain termination method (13) using Taqtrack™ sequencing kit (Promega, Madison, WI). Computer analysis of the sequence data was performed on a Macintosh IICX computer using the MacVector program from IBI (New Haven, CT).

Northern Blot Analysis. Total RNAs (10 μg/lane) were electrophoresed on 1.2% agarose gels in the presence of formaldehyde (2.2 mM) and transblotted onto a Nytran membrane(Schleicher & Schuell, Keene, NH). The hybridization was performed as described previously (11,12).

Nuclear Run-on Transcription Assays. Nuclei isolation and *in vitro* transcription reaction were performed as described previously (12). 5 μg each of the MT78, b-actin and pUC19 DNA was denatured in 0.4 N NaOH at 65°C for 10 min, then neutralized and blotted onto Nytran membranes using a slot-blotter (Bio-Rad, Richmond, CA). Hybridization was performed as described for Northern analysis.

RESULTS

cDNA Cloning of the Monkey Thioredoxin Gene. A differential screening method was used to isolate vitamin A-responsive clones from a monkey TBE cDNA library. One of the isolated clones, MT78, which was consistently stimulated by vitamin A, was studied further. The identity

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GAA TTC GCT TTG GAT CCA TTT CCA TCG GTC CTT ACA GCC GCT 42

                            1               5
CCT CAG ACC CCA GCA GCA AAG ATG GTG AAG CAG ATC GAG AGC 84
                            M   V   K   Q   I   E   S

        10                  15                  20
AAG GCT GCT TTT CAG GAA GCC TTG GAC GAC GCA GGT GAT AAA 126
K   A   A   F   Q   E   A   L   D   D   A   G   D   K

            25                  30                  35
CTT GTA GTA GTT GAC TTC TCA GCC ACG TGG TGT GGG CCT TGC 168
L   V   V   V   D   F   S   A   T   W   C   G   P   C

                40                  45
AAA ATG ATC AAG CCT TTC TTT CAT TCC CTC TCT GAA AAG TAT 210
K   M   I   K   P   F   F   H   S   L   S   E   K   Y

50                  55                  60
TCC AAC GTG GTA TTC CTT GAA GTA GAT GTG GAT GAC TGT CAG 252
S   N   V   V   F   L   E   V   D   V   D   D   C   Q

    65                  70                  75
GAT GTT GCT TCA GAG TGT GAA GTC AAA TGC ATG CCA ACA TTC 294
D   V   A   S   E   C   E   V   K   C   M   P   T   F

        80                  85                  90
CAG TTT TTT AAG AAG GGA CAA AAG GTG GGT GAA TTT TCT GGA 336
Q   F   F   K   K   G   Q   K   V   G   E   F   S   G

            95                  100                 105
GCC AAT AAG GAA AAG CTT GAA GCC ACC ATT AAT GAA TTA GTC 378
A   N   K   E   K   L   E   A   T   I   N   E   L   V

TAA TCA TGT TTT CTG AAA ACA TAA CCA GCC ATT GGC TAT TTA 420
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AAA CTT GTA ATT TTT TTA ATT TAC AAA AAT ATA AAA TAT GAA 462

GAC ATA ACC AGT TGC CAT CTG CGT GAC AAT AAA CAT TAT GCT 504

AAn
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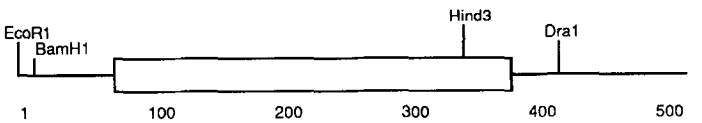

Fig. 1. Nucleotide sequence and restriction enzyme map of monkey thioredoxin cDNA. The polyadenylation signal (AATAAA) is underlined. The four amino acid active site (CGPC) is shown in bold letters. The open box in the restriction map represents the open reading frame.

of MT78 was determined by nucleotide sequencing. The restriction sites and nucleotide sequence of MT78 are shown in Fig. 1. The size of the MT78 cDNA is 506 nucleotides. A computer search of GenBank revealed that MT78 shared 97% homology with the human thioredoxin cDNA (14). The cDNA insert of the MT78 clone contains the whole open reading frame of 105 amino acids (315 nucleotides), the 3'-untranslated region (128 nucleotides), and the 5'-untranslated region (63 nucleotides) of the thioredoxin gene (Fig.1). The deduced amino acid sequence of monkey thioredoxin shows homology with the thioredoxin of human (95.2%), rabbit (90.4%), rat (89.5%), chicken (73.0%), yeast (47.6%) and E. coli (22.3%). A complete homology was found in the four amino acid sequence (CGPC), which is the active site among all the organisms compared.

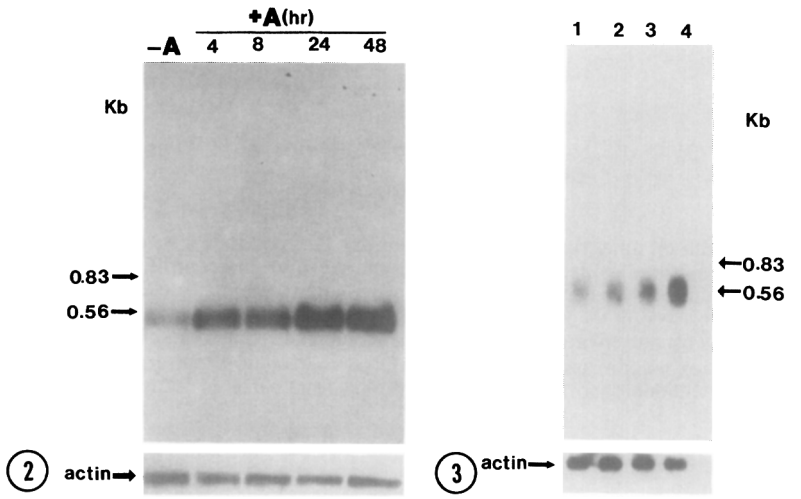

Fig. 2. Time-course effects of vitamin A on the thioredoxin mRNA level. Monkey TBE cells were cultured in the serum-free hormone-supplemented medium in the absence of vitamin A. At day 7, retinol (1 mM) was added and total RNAs were isolated at various times thereafter as indicated from these cultures. b-actin was used as a control. –A: without vitamin A; +A: with vitamin A.

Fig. 3. Effect of cycloheximide on the thioredoxin mRNA level. Monkey TBE cells were cultured as described in Fig. 2. Cycloheximide was added at the same time retinol was added (zero hour). Cultures were harvested 24 h later for RNA isolation. b-actin was used as a control. Lane 1: Without vitamin A and cycloheximide; lane 2: Without vitamin A and with cycloheximide; lane 3: With vitamin A and without cycloheximide; lane 4: With vitamin A and cycloheximide.

Regulation of the Thioredoxin Gene Expression by Retinol in Monkey TBE Cells. The effect of retinol on the expression of the thioredoxin gene in monkey TBE cells was studied by Northern blot analysis. As shown in Fig. 2, the level of thioredoxin mRNA was relatively low in monkey TBE cells cultured without retinol, and increased significantly after treating with retinol for 4 h. After 48 h, the level of thioredoxin had increased a maximal 10 fold. The level of the b-actin mRNA control did not change significantly during this period. Since the stimulation by retinol was observed after only 4 h, we wanted to determine whether or not this stimulation required new protein synthesis. Cycloheximide, an inhibitor of protein synthesis, was added simultaneously with retinol to the culture. We observed that cycloheximide superinduced the thioredoxin message (Fig. 3); however, the stimulation caused by retinol was relatively unchanged. To further understand the regulation process, we used a nuclear run-on assay to investigate whether or not this stimulation occurred at the transcriptional level. As shown in Fig. 4, the retinol treatment resulted in an 8-10 fold increase in the run-off thioredoxin transcription in nuclei. The control b-actin transcription was unchanged and the run-off transcripts were not hybridized to the plasmid DNA, pUC19.

DISCUSSION

Thioredoxin is a very conserved protein and is found in every organism. Using the differential hybridization technique, a cDNA clone of monkey thioredoxin gene was isolated in this

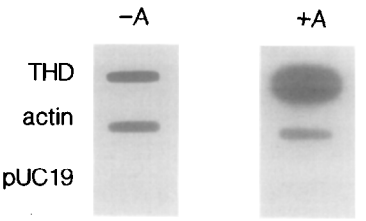

Fig. 4. Nuclear run-on transcriptional assay of monkey TBE cells after retinol treatment. Monkey TBE cells were cultured as described in Fig. 2. Nuclei from the control (without retinol) and retinol-treated cultures were harvested after 48 h of retinol treatment,. Nuclear run-on assays were carried out as described in the text. An equal amount of radioactivity of run-off transcripts from both cultures was used to hybridize nitrocellulose membranes containing cDNA inserts of MT78 (THD, thioredoxin), b-actin, and plasmid pUC19 DNA. -A: run-off transcripts from nuclei of the control cultures (without retinol); +A: run-off transcripts from nuclei of retinol-treated cultures.

study. The monkey thioredoxin contains the same 105 amino acids as other thioredoxins reported in vertebrates (14-16). It also possesses the consensus sequence Cys-Gly-Pro-Cys, which is found at the active sites of all thioredoxins from bacteria to human. When this deduced amino acid sequence is compared with sequences derived from other species, the monkey thioredoxin showes different homologies to human (95.2%), rabbit (90.4%), rat (89.5%), chicken (73.0%), yeast (47.6%), and E. coli (22.3%) (data not shown). The sequence homology of thioredoxins from different organisms correspond nicely to the evolutionary positions of these species.

Vitamin A has been demonstrated to be a potent regulator for the differentiation of respiratory epithelial cells (1-5). One of the underlying mechanisms could be to affect the expression of certain genes, which, in turn, could modulate the expression of other genes or modulate the functions of other proteins. In the present study, we have shown that retinol significantly enhanced the expression of thioredoxin gene in monkey TBE cells. Based on three observations, we suggest that the thioredoxin gene may be one of the primary retinol-responsive genes. First, the enhanced expression of thioredoxin gene was detectable within only 4 h of retinol treatment. Next, the enhanced expression was a primary event and an intermediate protein synthesis step was not required. Finally, the mRNA level was enhanced due to an increase in the transcription rate.

In recent years, there is increasing evidence that the regulation by thioredoxin may be an important general mechanism controlling transcriptional factors (6, 8), perhaps comparable to protein phosphorylation (17). Thioredoxin has been shown to modulate the DNA binding activity of the Fos-Jun heterodimer, suggesting that the transcriptional activity mediated by AP-1 binding factors may be regulated by a thioredoxin redox mechanism (6). Thioredoxin has also been proven to be an endogenous glucocorticoid receptor activating factor (7). Recently, thioredoxin was identified as a gene that can modulate the interferon-g (IFN-g)-mediated growth arrest of HeLa cells (8). All this evidence suggests that thioredoxin is a versatile protein that can modulate many biophysical processes. We still do not know how the enhanced expression of the thioredoxin gene by retinol is related to TBE cell differentiation. One possibility is that the enhanced expression of the thioredoxin gene may result in changes of expression of many other genes or changes of functions of other proteins, including those functioning in signal transduction pathways, and finally lead to the differentiation of TBE cells.

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