Increased Collagen Biosynthesis and Increased Expression of Type I and Type III Procollagen Genes in Tight Skin (TSK) Mouse Fibroblasts

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The Tight Skin (TSK) mouse is a mutant strain that displays connective tissue abnormalities characterized by excessive accumulation of collagen in skin, subcutaneous tissues, and some internal organs such as the heart. Increased collagen biosynthesis by skin organ cultures from affected mice has been previously demonstrated, but the mechanisms responsible have not been identified. In order to examine the molecular alterations responsible for this increased production of this protein, normal and TSK mouse dermal fibroblast cell lines were established, and studies of collagen biosynthesis and expression of Types I and III procollagen genes were performed. Secondary cultures of 5 normal and 5 TSK mice dermal fibroblasts were incubated in media containing 10% fetal calf serum and 50 µg/ml ascorbic acid and after labeling with [14C]proline for 72 h the amount of [14C]hydroxyproline synthesized was determined. The results showed that TSK mice dermal fibroblasts produced significantly greater amounts of [14C]hydroxyproline than their normal counterparts (118 ± 28.3 × 10⁻² versus 53.7 ± 21.9 × 10⁻² dpm/µg of DNA; p < 0.004). Subsequently, the expression of three procollagen genes in normal and TSK mice fibroblasts was analyzed by Northern blot hybridization of polyadenylated RNA to the human cDNA clones a12, Hf 32, and RJ 5 which are specific probes for transcripts of a1(I), a2(I), and a1(III) procollagen chains, respectively. It was found that TSK mice fibroblasts consistently displayed increased levels (up to 5-fold) of all three collagen transcripts while β-actin mRNA levels were unchanged. The results demonstrate that TSK mouse dermal fibroblasts produce excessive amounts of collagen in culture concomitant with a dramatic increase in the expression of Types I and III procollagen genes.

Under normal conditions, a strictly regulated balance is maintained between the amount of collagen synthesized by fibroblasts and the total concentration of this protein in the extracellular matrix. Normal fibroblasts are capable of modulating collagen synthesis according to the dynamic requirements of the extracellular matrix during development, differentiation, and repair. Although the intimate mechanisms responsible for this regulation are not known, there is increasing evidence to indicate that control of collagen gene expression is a complex process which may occur at transcriptional and post-transcriptional levels (1, 2). It has been suggested that coordinate regulation of collagen gene expression at the transcriptional level may reside in the conservation of certain regulatory nucleotide sequences in some of the collagen genes (3) and that differing steady-state mRNA levels may be primarily determined by differing rates of transcription of these genes (3–9).

Although there are several studies describing decreases in the expression of one or more collagen genes in various tissues, during developmental stages, or in certain pathological conditions (6, 10–13), there is a paucity of information regarding increased expression of one or more of the collagen genes at the transcriptional or post-transcriptional levels. Most of the studies performed to examine the mechanisms responsible for increased collagen gene expression have been performed employing fibroblasts obtained from patients with scleroderma, a disease characterized by the excessive deposition of collagen in skin and various internal organs. In one report, skin fibroblasts from two patients with scleroderma demonstrated a 2–7-fold increase in pro-α1(I) mRNA levels compared to normal controls (14). Another study (15) has demonstrated an increase in translatable procollagen mRNA in sclerodermic fibroblasts, suggesting an increase in the level of Type I procollagen mRNA. Because of the variability in disease stage at the time of fibroblast cell line establishment and the possible heterogeneity in the pathogenetic mechanisms responsible for the excessive collagen accumulation in scleroderma (16), studies of the molecular mechanisms responsible for increased expression of collagen genes employing sclerodermic fibroblasts may not yield conclusive results. In order to avoid these potential difficulties, we have examined fibroblasts from the TSK mouse, an animal model which displays a genetically determined increase in levels of collagen production (17) for increased levels of various procollagen mRNAs. Our results demonstrate that the increased synthesis of collagen in TSK mouse fibroblasts is accompanied by a striking elevation in the levels of Types I and III procollagen mRNAs. These findings may be primarily due to overtranscription resulting from a regulatory defect common to several collagen genes.

MATERIALS AND METHODS

Animals—Six-month-old TSK heterozygote mice were obtained from the Jackson Research Laboratories and were maintained on a...
regular Purina chow diet for 2-3 days before experiments were performed. Paired animals from the same strain born at the same time were used as normal controls. Most of these were litter mates of the TSK mice. The animals were put to death with ether, and their skins were carefully removed and cleaned by blunt dissection after incision along the incision lines. The subcutaneous fat and dermal tissue were minced and used to obtain fibroblastic cell lines.

Establishment of Fibroblastic Cell Lines from TSK and Normal Mice Skin—In preliminary experiments, we found that the method of obtaining cultures of dermal fibroblasts from skin explants anchored at the bottom of a culture dish usually employed in our laboratories (18) did not lead to any observable outgrowth of fibroblasts. Subsequently, we found that addition of epidermal growth factor to the culture media of the explants resulted in a satisfactory outgrowth of fibroblasts; however, from 12 to 16 weeks were required before the cultures reached confluency. This is in contrast with human dermal fibroblasts which grow to confluency from the explants in 3-4 weeks.

In attempts to improve the establishment of fibroblast cell lines from normal and TSK mice skin, we utilized an enzymatic digestion method to obtain isolated fibroblasts before seeding into culture dishes. For this purpose, skin minces (2-3 mm³) were incubated with a mixture of bacterial collagenase/dispase (Boehringer Mannheim; collagenase, 0.1 unit/ml; dispase, 0.8 unit/ml) in 5 ml of Dulbecco's MEM. The skin minces were energetically shaken at 37 °C in 95% air, 5% CO₂ for 4 h. The solubilized material was carefully removed into a plastic tube containing MEM and 10% FCS. The entire procedure was repeated for an additional 3 h with the remaining tissues, and the solubilized material from both digestions was centrifuged for 15 min at 600 X g. The pellets were gently resuspended in MEM containing 10% FCS, and samples of the pellets, the supernatants, and the residual tissues were layered on tissue culture dishes. Only the pellets from the second enzymatic digestion yielded adequate monolayer cultures which could be maintained for several passages. All experiments were performed with low passage (2-8) cell lines to avoid the possibility of transformation during long-term cultures.

Collagen Synthesis by Cultured TSK and Normal Mouse Dermal Fibroblasts—To examine the produced collagen, normal and TSK mice dermal fibroblasts, secondary cultures of 5 normal and 5 TSK mice were initiated from one T-175 flask of each cell line at passage 5. Each flask was trypsinized, and the harvested and counted. Approximately 3 x 10⁶ cells were then seeded into 35-mm dishes in triplicate and were incubated at 37 °C in MEM containing 10% FCS, 100 units/ml penicillin, and 50 µg/ml streptomycin. The culture media were changed three times a week and when the cells were approaching confluence the media were supplemented with 50 ng/ml ascorbic acid.

To examine collagen biosynthesis the confluent cells were labeled with 0.5 µCi of [³⁵S]proline/dish in media containing 10% FCS, 50 µg/ml ascorbic acid, and antibiotics for a period of 72 h. At the end of the labeling period, the media were removed and placed in an ice bath, and 0.25 ml of a stock solution containing protease inhibitors was added to give a final concentration of 5 mM EDTA, 5 mM N-ethylmaleimide, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM p-aminothiazol HCl. The cells were washed twice with MEM containing 10% FCS and then they were removed from the dishes with a rubber policeman. The suspended cells were transferred into culture plastic tubes and resuspended in MEM containing 10% FCS, and the stock protease inhibitor mixture and aliquots of cell suspensions were used to determine DNA content employing a diamine method (19). To determine the amount of [³⁵S]proline incorporated by the cultures, samples of media and cell suspensions were denatured at 100 °C with 1% SDS in the presence of 0.1% mercaptoethanol for 3 min and dialyzed against 0.1 M phosphate-SDS buffer. The dialysis was continued until the dialysate reached background radioactivity. Aliquots of the dialyzed media and cell extracts were used to determine total [³⁵S]proline incorporation employing a standard scintillation cocktail. Separate aliquots of media and cell extracts were hydrolyzed in 6 N HCl at 110 °C for 18 h after dialysis and subsequent 30% HCl/0.02% formaldehyde (31). The samples were heated to 65 °C for 2 h, and prehybridized in a solution containing 0.8% agarose, 20 mM Mops, pH 7.0, and 2.2 mM formaldehyde (31). The RNA was then transferred to nitrocellulose filters (32), dried in vacuo at 80 °C for 2 h, and prehybridized in a solution of 50% formamide, 0.1% SDS, 2 X SSC, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 50 µg/ml sonicated salmon sperm DNA for 4 h at 45 °C. The filters were then hybridized in the above solution containing 7 ng/ml [³²P]-labeled procollagen cDNA clones (>2 x 10⁶ cpm/ml). The sizes of the mouse poly(A) RNAs hybridizing to the procollagen cDNA probes were roughly equivalent to those seen in human poly(A) RNA.

Northern Blots—Samples of poly(A) RNA from control and TSK fibroblasts were made 50% formamide, 2.0 M in formaldehyde and 20 mM in Mops. The samples were heated to 65 °C for 5 min before being subjected to electrophoresis at 70 °C in 20 X SSC for 1 hr and then once in 0.25 X SSC for 10 min at 80 °C and once in 0.1 X SSC for 10 min at 80 °C. Each sample was run through 0.25% agarose, 20 mM Mops, pH 7.0, and 2.2 M formaldehyde (31). The RNA was then transferred to nitrocellulose filters (32), dried in vacuo at 80 °C for 2 h, and prehybridized in a solution of 50% formamide, 0.1% SDS, 2 X SSC, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 50 µg/ml sonicated salmon sperm DNA for 4 h at 45 °C. The filters were then hybridized in the above solution containing 7 ng/ml [³²P]-labeled procollagen cDNA clones (>2 x 10⁶ cpm/ml). After hybridization, the filters were washed two times in 2 X SSC for 15 min at room temperature, then once in 2 X SSC for 10 min at 68 °C, once in 1 X SSC for 10 min at 68 °C, and finally once in 0.1 X SSC for 10 min at 68 °C. The filters were air dried and exposed to x-ray film with intensifying screen at -70 °C for 2-5 days. Northern blots of control and TSK poly(A) RNA hybridized to procollagen cDNA probes were exposed for variable lengths of time for optimal visualization and densitometrically scanned on a Joyce-Loebl densitometer.

dot Blots—Samples of poly(A) mRNA from control and TSK fibroblasts were serially diluted in 200 µl of 20 % XSS and blotted in a dot manifold onto nitrocellulose paper which was pre-equilibrated with 20 % XSS. The wells were washed with 20 % XSS, and the dot blots were then dried in vacuo at 68 °C for 2 h, then prehybridized and hybridized as described above. The [³²P]-labeled β-actin cDNA was used to obtain a concentration of 5 ng/ml hybridization solution to give approximately 2 x 10⁶ cpm/ml. After hybridization, the dot blots were washed as described above, air dried, and exposed to x-ray film with intensifying screen at -70 °C for 4 days. The dots were densitometrically scanned on a Joyce-Loebl densitometer.

The abbreviations used are: MEM, minimal essential medium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; Mops, 3-(N-morpholino)propanesulfonic acid; kb, kilobase pair(s).
RESULTS

Incorporation of $[^{14}C]$Proline and Production of $[^{14}C]$Hydroxyproline by Normal and TSK Mice Dermal Fibroblasts—Total $^{14}C$ incorporation expressed on a per DNA basis in 5 normal and 5 TSK mice cell lines is shown in Table I. The average total $^{14}C$ incorporation was more than twice in TSK mouse fibroblast cultures compared to normal fibroblasts. This difference was highly significant ($p = 0.019$). The increment in $[^{14}C]$proline incorporation was entirely accounted for by newly synthesized proteins secreted into the media since no significant differences were found in the cell layer-associated radioactivity.

When $[^{14}C]$hydroxyproline production was analyzed, it was found that greater than 2-fold more $[^{14}C]$hydroxyproline was synthesized by the TSK cell lines than by the normal cell lines (Table II). Individually and as a group, each of the 5 TSK cell lines showed values higher than the highest $[^{14}C]$hydroxyproline value for the normal fibroblasts ($p < 0.004$). No differences were apparent in $[^{14}C]$hydroxyproline content of the cell layers between control and TSK mice fibroblast cultures.

When the relative distribution of radioactivity in media and cell layers was examined, it was found that the TSK mice cell lines secreted a greater proportion of newly synthesized proteins into the media than the normal cell lines 83 versus 63%. A comparison of the relative amounts of $[^{14}C]$hydroxyproline in media and cell layers also showed that most of the collagen synthesized in TSK mice cell lines was in the media (95 versus 80%).

Gel Electrophoresis of Media and Cell Layer Proteins from Control and TSK Fibroblast Cultures—To determine if there were differences in the populations of newly synthesized proteins between control and TSK fibroblasts, the labeled proteins present in the media and in the cell layers from representative cultures were analyzed by polyacrylamide slab gel electrophoresis in the presence of SDS (Fig. 1). When equal volume aliquots of media from each culture were examined, a higher proportion of radioactivity migrating in the region of collagenous polypeptides was detected in the media from the TSK mice cultures. There were no appreciable qualitative differences in the populations of labeled proteins synthesized by the two cell lines. It was of interest to note that in both cases, a large proportion of radioactivity appeared in the region of fully processed $\alpha$-collagen chains suggesting that in contrast to cultures of chick embryo or human dermal fibroblast cells, the cultured mouse fibroblasts were capable of more efficient processing of procollagen precursors to $\alpha$-chains. Electrophoretic analysis of the cell-associated labeled proteins did not reveal qualitative or quantitative differences between normal and TSK mice fibroblast cultures.

Expression of Collagen mRNAs in TSK Fibroblasts—Expression of three collagen genes in control and TSK fibroblasts was analyzed by Northern blot hybridization of polyadenylated RNA. RNA, selected by oligo(dT)-cellulose chromatography, was electrophoresed under denaturing condi-

### Table I

<table>
<thead>
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<th>Cell line</th>
<th>Media</th>
<th>Cell layers</th>
<th>Total $^{14}C$</th>
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<td>TSK</td>
<td>Normal</td>
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<tr>
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### Table II

<table>
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<td>Normal</td>
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<tr>
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<td>113.7 ± 29.4</td>
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<tr>
<td>$p$</td>
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<td>&lt;0.326</td>
<td>&lt;0.004</td>
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FIG. 1. SDS-polyacrylamide slab gel electrophoresis of newly synthesized proteins from media and cell layers of control and TSK mice fibroblast cultures. Equal aliquots (0.1 ml) of media and cell layer extracts from control and TSK mouse fibroblast cultures were electrophoresed under reducing conditions in the presence of SDS as described under “Materials and Methods.” Lane 1, standard chick tendon Type I procollagen and collagen; lane 2, media from TSK fibroblasts; lane 3, media from control mouse fibroblasts; lane 4, cell layer extract from TSK fibroblasts; lane 5, cell layer extract from control fibroblasts.

FIG. 2. Northern blot analysis of control (C) and TSK mice fibroblast poly(A') RNA hybridized to cDNA probes for α1(I), α2(I), and α1(III) procollagens. Poly(A') RNA was obtained from control and TSK mice fibroblasts as described under “Materials and Methods,” and equal amounts (4.0 μg) were loaded into each track. After electrophoresis, Northern blot hybridization was performed utilizing the cDNA probes α12, H32, and RJ5 which are specific for α1(I), α2(I), and α1(III) procollagens, respectively. Specific activities of the α12, H32, and RJ5 probes were 3.0 × 10⁴ cpm/μg, 4.8 × 10⁴ cpm/μg, and 3.5 × 10⁴ cpm/μg, respectively. Size markers are extrapolated from standard phage DNA restricted with HindIII.

TABLE III

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<th>Procollagen probe</th>
<th>Control fibroblasts</th>
<th>TSK fibroblasts</th>
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<td>α1(I)</td>
<td>4.7</td>
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<td>α2(I)</td>
<td>8.4</td>
<td>41.2</td>
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<td>α1(III)</td>
<td>5.4</td>
<td>30.1</td>
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DISCUSSION

Fibroblast collagen production is a complex process which under normal conditions is strictly regulated in order to maintain adequate concentrations of this protein in the extracellular matrix during steady state tissue remodeling as well as during more dynamic events such as differentiation, development, and repair. Despite the importance of collagen regulation in the maintenance of extracellular matrix integrity, the intimate mechanisms involved have not been completely elucidated. We previously demonstrated that the TSK mice mutant displays a genetically determined abnormality in collagen metabolism that results in excessive accumulation of this protein in skin and subcutaneous tissues (17). A similar
increase in collagen content in some internal organs of the affected mice has also been described (36). Studies of collagen biosynthesis in TSK mouse skin organ cultures demonstrated significantly increased production of collagen in comparison to normal mice skin (17). These results suggested that the TSK mutant mice may be an ideal system to examine the mechanisms responsible for regulation of collagen production at a molecular level. The results described in this paper demonstrate that dermal fibroblasts established from TSK mice maintain the phenotypic abnormality in collagen production during several passages in culture. As shown in Tables I and II, TSK mice fibroblasts produced greater than 2-fold more [14C]hydroxyproline than normal cells. Electrophoretic analysis of the newly synthesized collagen (Fig. 1) did not show detectable differences in the biosynthetic products of the TSK mice fibroblasts indicating that the mutation exclusively affected the mechanisms responsible for modulation of the amount of collagen produced by the cells. Because studies in a variety of other systems (6-13) have suggested that the primary site of control of collagen production resides at a transcriptional level, we performed experiments to examine if the increased production of collagen displayed by TSK mice fibroblasts results from alterations in the transcriptional control of collagen gene expression. When we analyzed the levels of mature procollagen-specific mRNA transcripts from normal and TSK mouse fibroblasts, we found that the TSK mouse fibroblasts, upon repeated experiments and with multiple samples, consistently demonstrated an approximate 5-fold increase in the levels of $\alpha_1$(I), $\alpha_2$(I), and $\alpha_1$(III) procollagen mRNA when compared with mRNA from normal sibling mice fibroblasts. There are a number of explanations to account for the higher levels of procollagen mRNAs in the TSK mice fibroblasts. For example, the increase in poly(A$^+$) RNA for $\alpha_1$(I), $\alpha_2$(I), and $\alpha_1$(III) in the TSK RNA population may be due to an accumulation of these mature transcripts resulting from an increase in procollagen mRNA stability rather than an actual increase in transcription in the collagen genes. Alternatively, the increased levels of the procollagen mRNAs may be due to a combination of increased transcription and decreased degradation rates. The latter situation has recently been reported to occur when primary avian tendon cells are treated with ascorbate in culture (37). This study demonstrated a 6-fold increase in $\alpha_2$(I) mRNA as a result of a 3-fold increase in the rate of transcription of the $\alpha_2$(I) procollagen gene and a 2-fold increase in the half-life of the $\alpha_2$(I) procollagen message. Studies are currently in progress in our laboratory to establish whether or not there is an increase in primary transcripts for the collagen genes studied here by in vitro transcription analyses. It is also possible that the overall transcriptional machinery of the TSK mice fibroblasts may be abnormal leading to overtranscription of many or all genes and/or abnormal processing of the primary transcripts. This possibility is not consistent with the phenotypic abnormality expressed by the TSK mouse; moreover, studies presented here with the noncollagenous cDNA probe $\beta$-actin suggest that this is not the case.

Studies on the transcription of procollagen genes by a number of investigators have suggested that in most, but not all, cases there is coordinate transcriptional regulation of collagen synthesis. For example, in chick cells infected with a temperature-sensitive mutant of the Rous sarcoma virus, levels of Type I procollagen mRNAs were shown to be low at the permissive temperature for src gene activation and elevated at the nonpermissive temperature (12). In this system, both pro-$\alpha_2$(I) and pro-$\alpha_1$(I) mRNA levels were decreased in the Rous sarcoma virus-transformed chick fibroblasts; however, the mechanism of this undertranscription of Type I procollagen mRNAs has not been identified. A more recent study has demonstrated that when transformed mouse cells are analyzed by dot-blot hybridization for levels of procollagen $\alpha_1$(III)- and $\alpha_2$(I)-specific mRNA, some transformants showed a coordinate decrease in expression of the two genes while another transformant demonstrated a dramatic increase in the level of $\alpha_1$(III) message with a concomitant decrease in the level of $\alpha_2$(I) message (38). Again, the mechanism responsible for this paradoxical finding is not yet clear. It has been suggested that in cases in which the levels of expression of collagen genes are singularly or coordinately altered, there may be some regulatory element(s) responsible for these alterations or some change in the conformation of chromatin around the collagen genes which influences activation of these genes (3, 39). For example, Raghow et al. (40) have reported that a "fibrogenic factor" isolated from thioacetamide-induced fibrotic rat liver specifically stimulates the synthesis of Type I collagen in cultured rat fibroblasts at the level of transcription. The mechanism of action of this factor is not understood at this time. As a result of the studies reported here, we suggest that increases in the levels of several procollagen mRNAs in the TSK fibroblasts may be due to a defect in a "regulatory element" which may affect the rates of transcrip-

### Table IV

<table>
<thead>
<tr>
<th>Poly(A$^+$) RNA µg</th>
<th>Control fibroblasts</th>
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tation of several members of the collagen gene family by a mechanism as yet unknown.

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REFERENCES