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Neena Philips
Thomas Jefferson University

Reza I. Bashey
Thomas Jefferson University

Sergio A. Jimenez
Thomas Jefferson University, Sergio.Jimenez@jefferson.edu

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Increased α1(I) Procollagen Gene Expression in Tight Skin (TSK) Mice Myocardial Fibroblasts Is Due to a Reduced Interaction of a Negative Regulatory Sequence with AP-1 Transcription Factor*

Neena Philips‡, Reza I. Bashey, and Sergio A. Jiménez§

From the Division of Rheumatology, Department of Medicine, and the Department of Biochemistry and Molecular Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

The TSK mouse, a model of fibrosis, displays exaggerated connective tissue accumulation in skin and visceral organs including the heart. To study the mechanisms of myocardial fibrosis in TSK mice, we established several strains of TSK mice myocardial fibroblasts in culture and examined the regulation of collagen gene expression in these cells. These strains displayed increased collagen gene expression in comparison with myocardial fibroblasts established from normal mice. On an average, the TSK myocardial fibroblast cultures showed a 4-fold increase in collagen synthesis and 4.4- and 3.6-fold increases, respectively, in α1(I) and α1(III) collagen mRNA steady state levels. The increased α1(I) and α1(III) collagen mRNA levels were mainly due to increased transcription rates (3.4- and 3.8-fold higher, respectively) of the respective genes. Furthermore, we showed that the up-regulation of α1(I) procollagen gene transcription in TSK mice myocardial fibroblasts was due to the lack of the strong inhibitory influence of a regulatory sequence contained in the promoter region encompassing nucleotides −675 to −804. Nuclear extracts from TSK mice myocardial fibroblasts showed lower DNA binding activity to oligonucleotides spanning the mapped regulatory sequence as well as to a consensus AP-1 sequence, but not to a consensus SP-1 sequence, and supershift experiments with an AP-1 antibody confirmed the interaction of these oligonucleotides with AP-1 protein.

These observations indicate that a strong negative regulatory sequence contained within −0.675 to −0.804 kilobase of the α1(I) procollagen promoter binds AP-1 transcription factor and mediates inhibition of gene transcription in normal murine myocardial fibroblasts. The TSK mice myocardial fibroblasts lack this inhibitory control, due to lower available amounts and/or decreased binding activity to this inhibitory sequence, and hence display increased α1(I) procollagen gene expression.

A prominent feature of fibrosis is the accumulation of excessive extracellular matrix components, especially types I and III collagens. The TSK mice mutation, which is characterized by

excessive collagen accumulation in tissues such as skin and heart, is a prototype for understanding the molecular pathogenesis of fibrosis (Green *et al.*, 1976; Jimenez *et al.*, 1986; and Osborn *et al.*, 1987). TSK mice skin organ cultures as well as TSK mice dermal fibroblasts display higher collagen synthesis, in comparison with normal (Jimenez *et al.*, 1984, 1986). The TSK mouse myocardium shows enhanced collagen accumulation within the interstitium (Osborn *et al.*, 1987), displays thicker collagen type I and collagen type III fibers and contains increased levels of α2(I), α1(III), and α2(IV) procollagen mRNAs (Chapman and Eghbali, 1990). Higher collagen biosynthetic activity, collagen type VI content, and α2(VI) collagen mRNA levels have also been demonstrated in organ cultures, tissue, and cultured fibroblasts of TSK mice hearts in comparison with normal (Bashey *et al.*, 1993). Here, we examined the expression of collagen types I and III in normal and TSK mice myocardial fibroblasts and determined the *cis*-acting regulatory elements governing transcriptional activity of an α1(I) procollagen promoter in normal and TSK mice myocardial fibroblasts. Furthermore, we examined the alterations in *trans*-acting factors responsible for the up-regulation of type I collagen expression in the TSK mice myocardial fibroblasts.

Overall, our observations imply that decreased binding of AP-1 transcription factor to a negative regulatory sequence between −0.675 and −0.804 kb[1] of the α1(I) procollagen promoter resulted in increased transcriptional activity of α1(I) procollagen gene which in turn led to enhanced procollagen mRNA levels and collagen biosynthesis in TSK mice myocardial fibroblast strains, in comparison with normal.

MATERIALS AND METHODS

Establishment of Normal and TSK Mice Myocardial Fibroblast Cultures—Hearts from six to eight heterozygous TSK (Tsk/+) mice (about 6 months old) and their normal littermates (pa/pa) were used for the establishment of each myocardial fibroblast strain. Four strains of myocardial fibroblasts were isolated, cultured, and passaged as described previously by Bashey *et al.* (1992). Briefly, minced myocardial tissue pieces were incubated in Hanks' buffer containing trypsin (0.1 µg/µl) and collagenase (50 units/ml) for 10 consecutive incubations of 10 min each at 37 °C. Cells in the supernatants from each of the digestions were pelleted, resuspended in culture media, and seeded on 100-mm tissue culture dishes for 3 h, after which the incubation media containing nonadherent cells was discarded and the plastic-attached cells were cultured. When the cultures reached confluence, the cells were dissociated with trypsin and subcultured as described previously (Bashey *et al.*, 1992).

Collagen Biosynthesis—Protein synthesis was determined by measuring total [^{14}C]proline incorporation in normal and TSK mice myocardial fibroblasts as described by Jimenez *et al.* (1986). Biosynthesis of [^{14}C]collagenase-sensitive proteins was measured by collagenase diges-

[1] The abbreviations used are: kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase.

TABLE I

Collagen biosynthesis by normal and TSK mice myocardial fibroblast strains

Fibroblast strains	Total [^{14}C]proline incorporation (cpm/μg DNA X 10^{-4})						TSK/N
	Media		Cells		Total		
	N	TSK	N	TSK	N	TSK	
2	12.1 ± 0.3	15.7 ± 0.7	18.1 ± 0.2	60.4 ± 2.8	30.2 ± 0.2	76.2 ± 2.8	2.5
3	5.1 ± 0.1	9.8 ± 0.2	14.5 ± 1.2	34.0 ± 0.3	19.6 ± 1.1	43.8 ± 0.2	2.2
4	6.8 ± 0.4	17.2 ± 0.8	18.8 ± 1.2	51.6 ± 2.4	25.6 ± 1.1	68.8 ± 1.8	2.7

Fibroblast strains	[^{14}C]Collagenase sensitive proteins (cpm/μg DNA X 10^{-3})						TSK/N
	Media		Cells		Total		
	N	TSK	N	TSK	N	TSK	
2	43.2 ± 1.5	30.5 ± 1.7	5.9 ± 0.3	83.1 ± 2.7	49.1 ± 1.3	113.7 ± 1.8	2.3
3	12.4 ± 0.9	22.8 ± 0.5	14.2 ± 1.6	67.8 ± 1.4	26.6 ± 1.3	90.6 ± 1.3	3.4
4	12.4 ± 0.8	41.5 ± 2.1	10.2 ± 0.3	85.6 ± 3.6	22.5 ± 0.7	127.1 ± 2.7	5.6

Passage 4 cultures of myocardial fibroblasts from normal (N) and TSK mice were labeled with [^{14}C]proline for 24 h. Media and cell layers were harvested, dialyzed, and total [^{14}C]proline incorporation determined by scintillation counting. Aliquots of media and cells were subjected to collagenase assay to determine [^{14}C]collagenase-sensitive proteins. Fluorometric DNA assays were performed to express data on a per DNA basis. Data from each strain is an average of triplicate cultures ± 1 standard deviation.

tion of the ^{14}C-labeled proteins exactly as described by Diegelmann and Peterkofsky (1972) and Peterkofsky and Diegelmann (1971). The [^{14}C]proline incorporation and [^{14}C]collagenase-sensitive proteins in media and cells were normalized for the DNA content of the cultures which was determined by DNA assays of aliquots of undialyzed cells as described previously by Labarca and Paigen (1980). To characterize the newly synthesized ^{14}C-labeled proteins, equal volume aliquots of media and cells were electrophoresed on 7% SDS-PAGE under reducing conditions as described by Bashey *et al.* (1992).

RNA Analysis—Confluent normal and TSK mice myocardial fibroblasts cultures in T-175 flasks were incubated with ascorbic acid (50 μg/ml media) for 24 h, and total RNA was extracted from harvested cells employing the guanidine/cesium chloride method (Sambrook *et al.*, 1989).

For Northern hybridization analysis of RNA, samples of total RNA were electrophoresed on 8% agarose, 2.2 M formaldehyde gels, transferred from the gels to nitrocellulose filters, and the filters were prehybridized as described previously (Jimenez *et al.*, 1986). The filters were hybridized at 45 °C overnight with [α-^{32}P]CTP-labeled murine α1(I) procollagen cDNA (French *et al.*, 1985), murine α1(III) procollagen cDNA (Liau *et al.*, 1985) and rat GAPDH cDNA (Marty *et al.*, 1985). The filters were washed under conditions of high stringency to 0.1 × SSC, 0.1% SDS at 65 °C and exposed to x-ray films overnight. The films were developed and scanned by densitometry.

Dot blot analysis was performed as described by Sambrook *et al.* (1989) and Davis *et al.* (1986). Serial dilutions of RNA prepared from each cell strain were made in loading buffer composed of 10 × SSC, to give final RNA concentrations from 3 to 0.7 μg or from 4 to 0.5 μg. The serial concentrations of RNA were dot-blotted and immobilized on nitrocellulose filters. The filters were prehybridized and hybridized with α1(I) procollagen, α1(III) procollagen, and GAPDH cDNAs as described above. The filters were washed under conditions of high stringency and exposed to x-ray films which were developed and scanned by densitometry. To correct for differences in RNA loading and transfer, the arbitrary densitometry units obtained from the hybridizations with α1(I) procollagen or α1(III) procollagen cDNAs were corrected for the arbitrary densitometric units obtained from hybridizations with GAPDH cDNA.

Analysis of Transcriptional Rates—The transcriptional rates of α1(I) procollagen and α1(III) procollagen genes in normal and TSK mice fibroblasts were determined by nuclear run-on assays (Sariban *et al.*, 1988). Equal numbers of normal and TSK mice myocardial fibroblasts were homogenized in homogenization buffer (0.3 M sucrose, 10 mM NaCl, 5 mM MgCl$_2$, 0.5 mM dithiothreitol, 10 mM Tris, pH 7.5, and 1% Triton X-100) and centrifuged at 2500 rpm at 4 °C for 5 min to pellet the nuclei. The pellets were then resuspended in nuclei storage buffer (40% glycerol, 50 mM Tris, pH 8.0, 5 mM MgCl$_2$, and 0.1 mM EDTA). The isolated nuclei were incubated with transcription mixture (90 mM KCl, 3 mM MgCl$_2$, 400 μM ATP, GTP, and UTP, 2 mM dithiothreitol, 1 unit/μl RNasin, and 4.1 μM [α-^{32}P]CTP) for 25 min at 25 °C. After proteinase K and DNase digestions the RNA was extracted with phenol/chloroform, precipitated, dissolved in hybridization solution, and the radioactivity of the samples determined by scintillation counting. Aliquots containing equal amounts of labeled RNA from normal and TSK mice fibroblasts were hybridized to α1(I)procollagen, α1(III) procollagen, and

GAPDH cDNAs immobilized on nitrocellulose filters. The filters containing the bound cDNAs were washed, exposed to x-ray films, and then counted in a liquid scintillation counter.

Analysis of Regulatory cis-Acting Elements—To determine whether there were differences in the transcriptional activity driven by various regulatory *cis*-acting elements in the α1(I) procollagen gene between normal and TSK mice myocardial fibroblasts, a series of human α1(I) procollagen promoter constructs (5′ end points from −5.3 kb to −0.08 kb) ligated to the CAT reporter gene prepared as described previously (Jimenez *et al.*, 1994) were transiently transfected into both types of cells. Plasmids were transfected into normal and TSK mice myocardial fibroblast strains by calcium phosphate/DNA coprecipitation, followed by glycerol shock for 1 min as described by Graham and Van Der Eb (1973). Forty-eight h after transfection, cell extracts containing equal amounts of protein were assayed for CAT activity by thin layer chromatography (TLC). Autoradiographs of TLC plates were scanned, and radioactive areas corresponding to acetylated and unacetylated chloramphenicol in the thin layer TLC plates were cut and counted in a liquid scintillation counter. The quantitative values obtained by scanning and scintillation counting were similar. To monitor for transfection efficiency, cells were cotransfected with pSV2AP, a plasmid containing a full-length alkaline phosphatase cDNA fused to the SV40 promoter and enhancer (Yoon *et al.*, 1988), and alkaline phosphatase activity in cell extracts was measured spectrophotometrically.

Analysis of trans-Acting Factors—Nuclear protein extracts were prepared from normal and TSK mice myocardial fibroblasts as described by Andrews and Faller (1991) and the protein content of the nuclear extracts was measured as described by Bradford (1976).

For mobility shift assays, three oligonucleotides spanning the mapped *cis*-acting sequence were synthesized. The oligonucleotides were end-labeled by incubating 100 ng of each of the oligonucleotides, with [γ-^{32}P]ATP (50 μCi), 1 × kinase buffer and T4 polynucleotide kinase (8 units) for 1 h at 37 °C. The end-labeled oligonucleotides were separated from unincorporated [γ-^{32}P]ATP by passing the mixture through a G-25 Sephadex column. For DNA binding reactions, 10 μg of nuclear extracts from normal or TSK myocardial fibroblasts were incubated with 1 × binding buffer (10 mM HEPES, pH 7.9, 60 mM KCl, 0.1 mM EDTA, and 1 mM dithiothreitol), 1 μg of poly(dI-dC), 1% glycerol, and 0.5 ng of labeled oligonucleotide on ice for 30 min in a total volume of 10 μl. Following incubation, the DNA-protein complexes were separated from free probe by electrophoresis on 4% polyacrylamide gels with 1 × Tris/glycine as running buffer. The gels were dried and exposed to x-ray films overnight. The autoradiographs obtained were scanned in a laser densitometer and the arbitrary densitometric units obtained were expressed as ratios of normal/TSK.

For supershift assays, 10 μg of nuclear proteins were incubated with c-*jun*/AP-1 antibody (Santa Cruz Biotechnology Inc.) for 5 h at 4 °C and then subjected to DNA binding reaction as described above.

RESULTS

Establishment of Normal and TSK Mice Myocardial Fibroblast Strains—In order to study the mechanisms responsible for the accumulation of collagen in myocardial fibrosis and to examine the regulation of collagen gene expression in myocar-
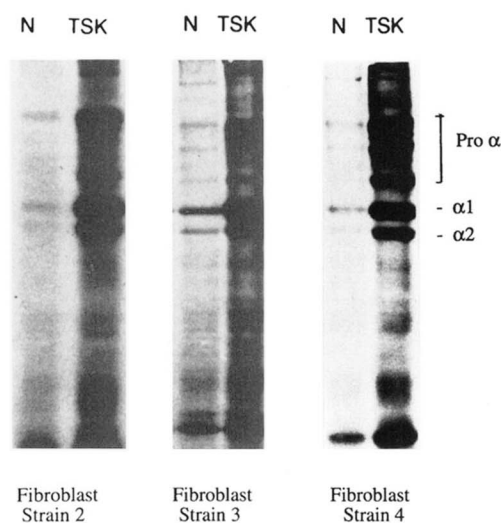
N TSK   N TSK   N TSK

Pro α
- α1
- α2

Fibroblast   Fibroblast   Fibroblast
Strain 2   Strain 3   Strain 4

FIG. 1. **SDS-PAGE analysis of ¹⁴C-labeled proteins in cell layers of normal (N) and TSK mice myocardial fibroblast strains.** Equal volume aliquots of labeled cell layers were electrophoresed on 7% SDS-PAGE under reducing conditions.

A   B
N TSK   N TSK

5.7 kb -
4.7 kb -

- 5.4 kb

GAPDH -

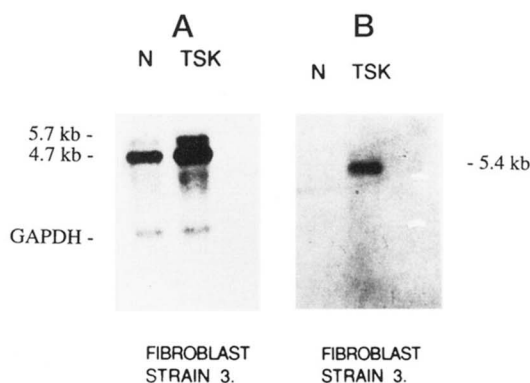FIBROBLAST   FIBROBLAST
STRAIN 3.   STRAIN 3.

FIG. 2. **Northern hybridization of total RNA from a normal and a TSK mice myocardial fibroblast strain.** Total RNA was electrophoresed on 8% formaldehyde-agarose gels, transferred to nitrocellulose filters, and the filters were prehybridized and hybridized with labeled cDNAs, washed, and exposed to x-ray film. *A*, hybridization with α1(I) procollagen and GAPDH cDNAs; *B*, hybridization with α1(III) procollagen cDNA.

TABLE II
Steady state levels of α1(I) and α1(III) procollagen mRNA in normal and TSK mice myocardial fibroblast strains

Total RNA from normal and TSK myocardial fibroblast cultures was examined by dot blot hybridizations as described under "Materials and Methods." Semiquantitative densitometric analysis of the blots following standardization with GAPDH mRNA levels in each RNA preparation was performed. The results are the averages of duplicate experiments and are expressed as a TSK/normal ratio.

Fibroblast strain	mRNA levels (TSK/normal)	
	α1(I) Procollagen	α1(III) Procollagen
1	2.5	3.0
2	2.0	2.5
3	4.0	4.5
4	9.0	4.5
Average	4.4	3.6

dial fibroblasts, we successfully isolated and cultured fibroblastic cells from normal and TSK mice myocardium. Four normal and four TSK mice myocardial fibroblast strains were established and are referred to as strains 1, 2, 3, and 4. Morphologically, fibroblasts derived from normal mice myocardium had characteristic elongated spindle shapes, multiple nucleoli, and agranular cytoplasm. Myocardial fibroblast cultures from TSK mice showed appreciable differences in morphology with most of the cells consistently displaying a star-shaped or rounded cell shape (not shown). The cultures established could be passaged serially for at least 12 passages without apparent changes in their morphology or in their patterns of growth and proliferation.

Collagen Biosynthesis by Normal and TSK Mice Myocardial Fibroblast Strains—Table I represents total [¹⁴C]proline incorporation and [¹⁴C]collagenase-sensitive proteins in media and cell layers of various normal and TSK mice myocardial fibroblast strains. In one TSK myocardial fibroblast strain the average total [¹⁴C]proline incorporation and [¹⁴C]collagenase-sensitive proteins, respectively, were 3.8- and 1.5-fold higher in passage-2 cultures and 3.7- and 5-fold higher in passage-8 cultures, in comparison with normal (not shown). The average total [¹⁴C]proline incorporation and [¹⁴C]collagenase-sensitive proteins, respectively, were 2.5- and 2.3-fold higher in a second TSK mice myocardial fibroblast strain, 2.2- and 3.4-fold higher in a third TSK mice myocardial fibroblast strain, and 2.7- and 5.6-fold higher in a fourth TSK mice myocardial fibroblast strain, in comparison with normal (Table I). In all four TSK mice myocardial fibroblast strains, there were much greater increases in the [¹⁴C]collagenase-sensitive proteins in the cell layers than in the media (Table I). On an average, the increases in [¹⁴C]proline-labeled proteins in media and cell layers, respectively, were 2- and 3.4-fold higher in TSK mice myocardial fibroblast strains, whereas the increases in [¹⁴C]collagenase-sensitive proteins in the TSK mice strains were 1.8-fold higher in media and 10-fold higher in cell layers, in comparison with normal. Furthermore, these differences in collagen biosynthesis were also reflected in SDS-PAGE autoradiograms which showed an increase in collagen in the media (not shown) but a much greater increase in cell layer-associated collagen in TSK fibroblast cultures, relative to normal (Fig. 1).

Steady State Levels of α1(I) Procollagen and α1(III) Procollagen mRNA in Normal and TSK Mice Myocardial Fibroblast Strains—The mRNA levels of types I and III procollagens were

analyzed to determine whether the increased collagen biosynthesis in TSK mice myocardial fibroblast cultures was due to increased collagen mRNA steady state levels. In comparison with normal, the TSK mice myocardial fibroblast strains showed increased α1(I) procollagen and α1(III) procollagen mRNA steady state levels as analyzed by Northern and dot blot hybridizations. As expected, two α1(I) procollagen transcripts corresponding to 5.7 and 4.7 kb were seen in Northern blots hybridized with α1(I) procollagen cDNA, whereas only 5.4-kb transcripts were found in the Northern blots hybridized with α1(III) procollagen cDNA (Fig. 2). The mRNA steady state levels of α1(I) procollagen and α1(III) procollagen were quantitatively analyzed relative to GAPDH, a non-collagenous constitutive protein, by scanning autoradiograms of Northern and dot blot hybridizations and standardizing procollagen mRNA levels with GAPDH mRNA levels in the respective RNA preparations (Table II). All the TSK myocardial fibroblast strains showed greater than 2-fold increases in types I and III procollagen mRNAs and in one cell strain (strain 4) the increases were 9.0- and 4.5-fold, respectively. The increased collagen mRNA steady state levels corresponded with the increased collagen biosynthetic activity of these TSK myocardial fibroblast strains, suggesting altered regulation of collagen gene expression in TSK mice myocardial fibroblast strains at a pretranslational level.

Transcription Rates of α1(I) and α1(III) Procollagens Genes in Normal and TSK Mice Myocardial Fibroblast Strains—Nuclear run-on assays using cDNAs for types I and III procollagens indicated that the increased mRNA levels for these
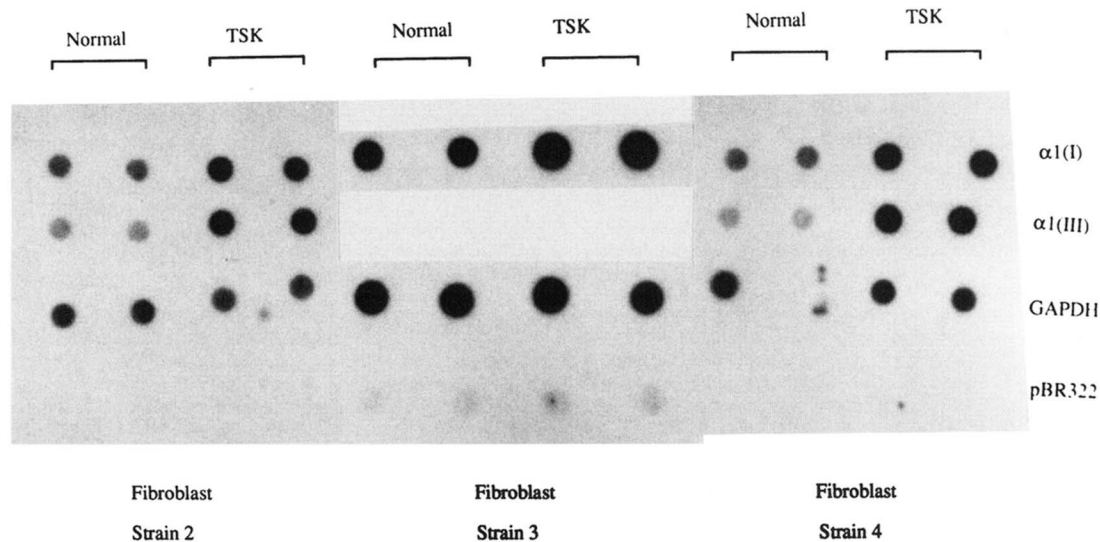
FIG. 3. **Transcriptional activities of α1(I) procollagen, α1(III) procollagen and GAPDH genes in normal and TSK mice myocardial fibroblast strains.** Initiated transcripts in nuclei isolated from normal and TSK mice myocardial fibroblast strains were elongated in the presence of [^{32}P]UTP. The radiolabeled transcripts were extracted from nuclei and hybridized to α1(I) procollagen, α1(III) procollagen, and GAPDH cDNAs immobilized on nitrocellulose filters. The filters were washed and exposed to x-ray films overnight.

proteins in cultured TSK mice myocardial fibroblasts were due to increased transcription of the respective genes (Fig. 3). In comparison with normal, the transcription rate of the α1(I) procollagen gene in TSK mice myocardial fibroblast strains 2, 3, and 4, following standardization for the transcription rate of the GAPDH gene was, respectively, 2.3-, 3.4-, and 4.4-fold higher. With the exception of strain 4, the observed increases in transcription rates of the α1(I) procollagen gene in these TSK mice myocardial fibroblast strains paralleled the increases in steady state α1(I) procollagen mRNA levels in the respective strains. Hybridization of labeled transcripts with α1(III) procollagen cDNA showed that the transcription rates α1(III) gene were 3.4- and 6.4-fold higher in TSK mice myocardial fibroblast strains 2 and 4, respectively, in comparison with normal. These values also largely corresponded with the observed increase in α1(III) procollagen mRNA levels in these strains. The transcription rate of GAPDH gene was fairly similar in normal and TSK mice myocardial fibroblast strains. Control hybridizations of the labeled transcripts from normal and TSK mice fibroblasts to filters containing the plasmid pBR322 showed only very faint radioactivity indicating the specificity of the α1(I) procollagen, α1(III) procollagen, and GAPDH cDNAs for their respective transcripts. The parallel increases in transcription rates of α1(I) procollagen and α1(III) procollagen genes in TSK mice myocardial fibroblast strains imply the involvement of common transcriptional regulatory mechanisms. Furthermore, since the increase in collagen biosynthesis in TSK mice myocardial fibroblast strains correlated with the increases in collagen mRNA steady state levels, which in turn correlated with increased transcription rates of procollagen genes, it can be concluded that the up-regulation of procollagen gene expression in TSK mice myocardial fibroblast strains is mediated transcriptionally.

Functional Analysis of Regulatory cis-Acting Elements of the Human α1(I) Procollagen Promoter in Normal and TSK Mice Myocardial Fibroblast Strains—A series of human α1(I) procollagen promoter-CAT constructs (5′ end points at −5.3, −2.3, −0.804, −0.675, −0.463, −0.369, −0.174, and −0.084 kb) were transiently transfected into TSK and normal mice myocardial fibroblast cultures to map the regulatory sequences responsible for the up-regulation of promoter activity in TSK fibroblast cultures (Fig. 4). The transfection efficiency was monitored by cotransfecting each of the procollagen promoter deletion con-

structs with the pSV2AP plasmid followed by the measurement of alkaline phosphatase activity in whole cell extracts. No significant differences were found in alkaline phosphatase activity between normal and TSK myocardial fibroblast strains, indicating that the transfection efficiency was similar in both types of cells (results not shown).

The results of these studies with strains 3 and 4 are shown in Table III and are illustrated in Fig. 4. Maximal differences in CAT activity between normal and TSK strains were consistently seen with the sequence from −0.804 to −0.675 kb, thus mapping this region as the principal region involved in the differential regulation of promoter activity between normal and TSK strains. Quantitative analysis of these results showed that in comparison with normal, the CAT activity of the −0.804 kb α1(I) procollagen promoter-CAT construct was 4.6-fold higher in TSK mice fibroblast strain 3 and 6.5-fold higher in TSK mice fibroblast strain 4. Since inclusion of the promoter sequence from −0.804 to −0.675 kb caused a reduction in CAT expression in normal fibroblast strains relative to the activity driven by shorter α1(I) procollagen promoter constructs, it can be inferred that this region is inhibitory. In contrast, inclusion of this promoter sequence (from −0.804 to −0.675 kb) did not alter CAT expression in TSK fibroblast strains, indicating that the inhibitory control exerted by this sequence in normal cells is absent in TSK strains. The slight increase in CAT expression (≈2-fold) with the −0.675- to −0.463-kb promoter construct in TSK strains suggests that the involved regulatory region may extend slightly further downstream from the −0.675-kb end point. The CAT activity driven by the shorter α1(I) procollagen promoter-CAT constructs, with 5′ end points at −0.463, −0.369, −0.174, and −0.084 kb, was similar in normal and TSK myocardial fibroblasts. Overall, the markedly higher CAT expression in the presence of the sequence from −0.804 to −0.675 kb α1(I) procollagen promoter in TSK mice myocardial fibroblast strains, in comparison with normal, provides strong evidence that the major regulatory elements responsible for the up-regulation of α1(I) procollagen gene expression in TSK mice myocardial fibroblast strains are located in this region.

Interaction of trans-Acting Factors in Nuclear Extracts from Normal and TSK Mice Myocardial Fibroblast Strains with the Mapped cis-Acting Regulatory Sequence—To examine the interaction of trans-acting factors with the regulatory region in the α1(I) procollagen promoter that showed maximal differ-
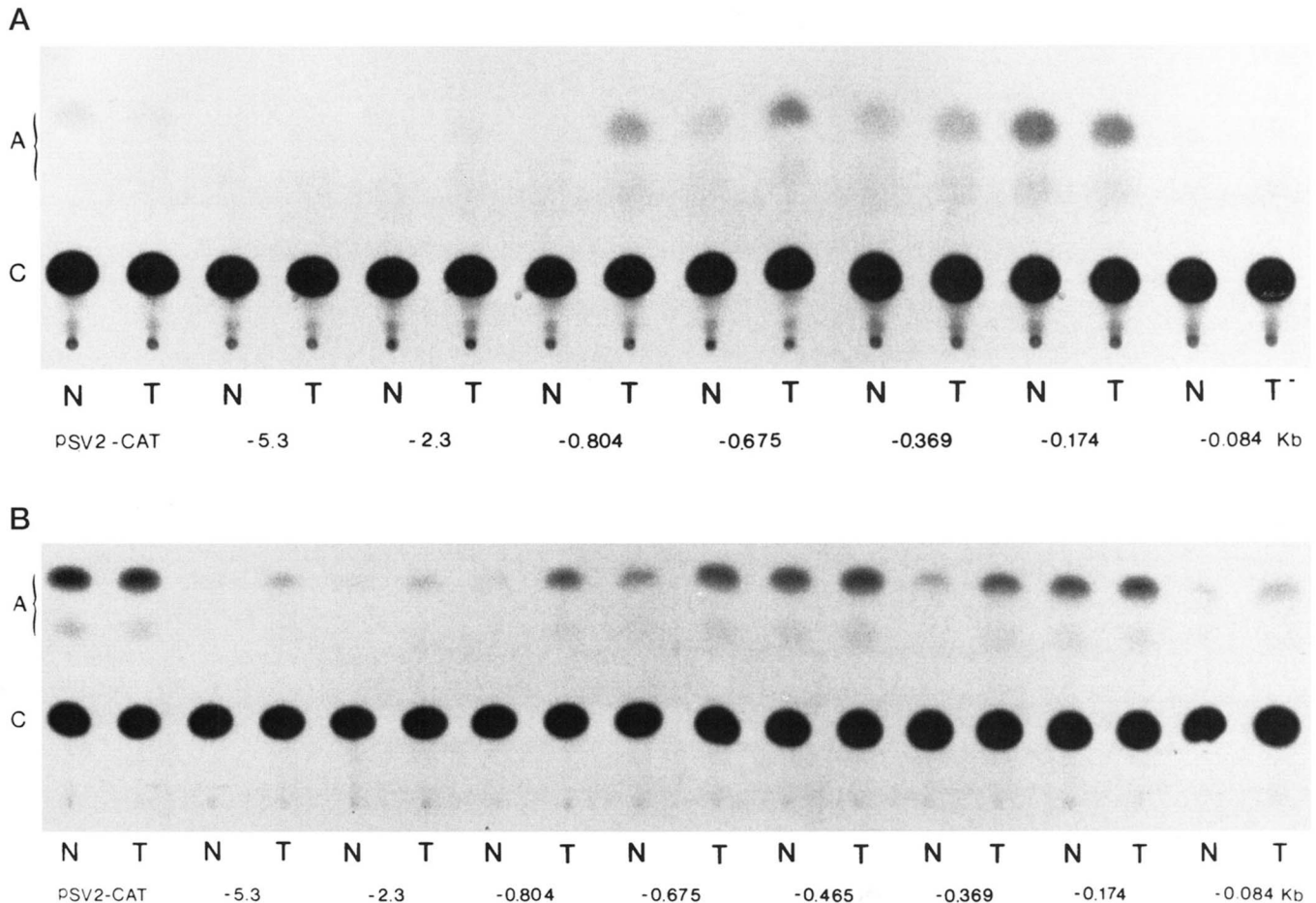
A

B

FIG. 4. **CAT activity of a series of α1(I) procollagen promoter-CAT constructs with 5′ end points from −5.3 to −0.08 kb, transfected into normal (N) and TSK (T) mice myocardial fibroblast strains.** α1(I) procollagen promoter deletion constructs and pSV2CAT plasmid, respectively, were transfected into normal and TSK mice myocardial fibroblasts by calcium phosphate precipitation, and cell extracts were assayed for CAT activity. The acetylated (A) and nonacetylated (C) chloramphenicol were separated by thin layer chromatography and exposed to x-ray film overnight at room temperature. A, strain 3; B, strain 4.

TABLE III

Quantitation of CAT activity of a series of α1(I) procollagen promoter-CAT constructs with 5′ end points from −5.3 to −0.084 kb, transfected into normal (N) and TSK mice myocardial fibroblasts

The autoradiograms of CAT assays corresponding to each strain were analyzed by densitometry and the areas corresponding to acetylated chloramphenicol were quantitated in arbitrary densitometric units (CAT activity). The CAT activity driven by each α1(I) procollagen construct was corrected for efficiency of transfection as described under "Materials and Methods," and the values obtained are shown. Comparison of expression of the various α1(I) procollagen promoter-CAT constructs in normal and TSK mice myocardial fibroblasts is represented as the ratio of TSK/normal. ND, not done.

Transfected plasmids	Strain 3			Strain 4			Average TSK/N
	N	TSK	TSK/N	N	TSK	TSK/N	
−5.3 α1(I)-CAT	1.9	2.8	1.5	5.4	21.9	4.0	2.75
−2.3 α1(I)-CAT	2.0	6.3	3.1	12.1	13.8	1.1	2.10
−0.8 α1(I)-CAT	2.8	13.0	4.6	7.6	49.5	6.5	5.55
−0.67 α1(I)-CAT	6.4	12.2	1.9	28.3	50.4	1.9	1.9
−0.46 α1(I)-CAT	ND	ND		43.4	51.7	1.1	1.1
−0.36 α1(I)-CAT	8.8	9.8	1.1	16.1	18.7	1.1	1.1
−0.17 α1(I)-CAT	16.2	15.0	0.9	55.7	47.7	0.9	0.9
−0.08 α1(I)-CAT	4.1	5.4	1.3	13.4	17.7	1.3	1.3

ences between normal and TSK fibroblast strains, DNA binding proteins present in the nuclei of normal or TSK myocardial fibroblasts that recognize sequences within this region were examined. For this purpose, end-labeled oligonucleotides 1, 2, and 3, which correspond, respectively, to sequences −0.804 to −0.763 kb, −0.762 to −0.718 kb, and −0.717 to −675 kb of the α1(I) procollagen gene (Fig. 5) were incubated with equal amounts of nuclear proteins from normal and TSK mice myocardial fibroblasts. As shown in Fig. 6, this interaction resulted in the formation of specific DNA-protein complexes. The DNA-protein complexes formed with nuclear extracts from normal and TSK mice fibroblasts and labeled oligonucleotides 1 (Fig. 6A), 2 (Fig. 6B), and 3 (Fig. 6C), respectively, displayed qualitatively similar electrophoretic mobilities. The labeled oligonucleotide-protein complexes formed were specific because their formation was not abolished by excess salmon sperm DNA, whereas it was abolished by excess amounts of the corresponding unlabeled oligonucleotide. Mobility shift assays showed that nuclear extracts from all TSK mice myocardial fibroblast strains contained much lower binding activity for each of the three oligonucleotides than nuclear extracts from normal mice fibroblasts (Table IV). On an average, in comparison with normal, the binding activities for oligonucleotides 1, 2, and 3 were, respectively, 3-, 3.7-, and 3-fold lower in TSK mice myocardial fibroblasts. These results suggest that the nuclear extracts from TSK mice fibroblasts contain lower binding activity for each of the three oligonucleotides.

Since all three oligonucleotides showed lower DNA-protein complex formation with nuclear extracts from TSK mice fibroblasts, in comparison with normal cells, and each contains transcription factor AP-1 binding sites, it seemed likely that each of the three oligonucleotides were bound by the same *trans*-acting factor. To examine this possibility, the ability of the oligonucleotides to compete with each other for DNA-protein complex formation was studied (Fig. 6). Oligonucleotides 1
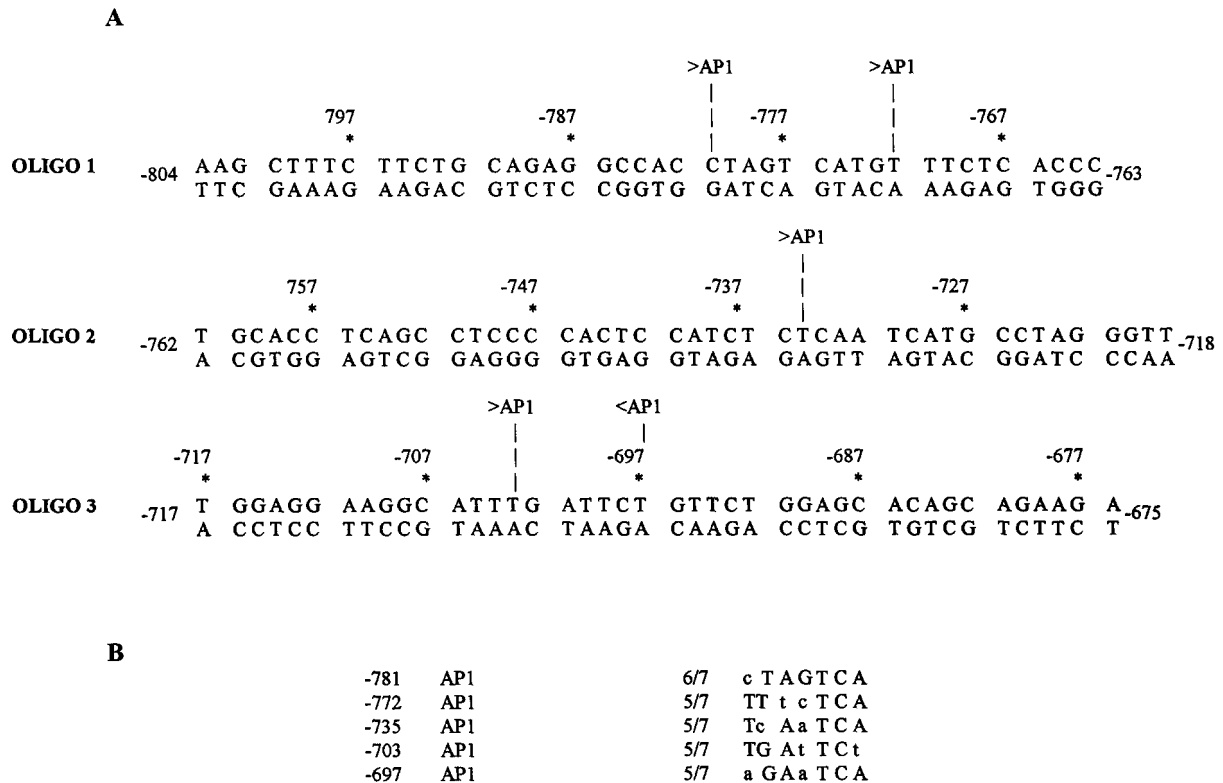
A

```
                                            >AP1              >AP1
                                             |                |
            797            -787              | -777           | -767
             *              *                |  *             |  *
OLIGO 1  -804 AAG CTTTC TTCTG CAGAG GCCAC CTAGT CATGT TTCTC ACCC -763
              TTC GAAAG AAGAC GTCTC CGGTG GATCA GTACA AAGAG TGGG

                                                   >AP1
                                                    |
            757           -747          -737        |    -727
             *             *             *          |     *
OLIGO 2  -762 T GCACC TCAGC CTCCC CACTC CATCT CTCAA TCATG CCTAG GGTT -718
              A CGTGG AGTCG GAGGG GTGAG GTAGA GAGTT AGTAC GGATC CCAA

                             >AP1     <AP1
                              |        |
           -717       -707    |      -697       -687        -677
             *          *     |        *          *           *
OLIGO 3  -717 T GGAGG AAGGC ATTTG ATTCT GTTCT GGAGC ACAGC AGAAG A -675
              A CCTCC TTCCG TAAAC TAAGA CAAGA CCTCG TGTCG TCTTC T
```

B

```
        -781   AP1      6/7   c TAGTCA
        -772   AP1      5/7   TT t c TCA
        -735   AP1      5/7   Tc Aa TCA
        -703   AP1      5/7   TG At TCt
        -697   AP1      5/7   a GAa TCA
```

FIG. 5. **Oligonucleotides used in mobility shift assays.** *A,* sequences of three oligonucleotides (1, 2, and 3, respectively) spanning the α1(I) procollagen promoter sequence from −804 to −675 base pairs. The AP-1 binding sites in these sequences are shown. *B,* homologies between the consensus AP-1 sequence and the AP-1 binding sites present within −804 to −675 base pairs of the α1(I) procollagen promoter.

and 3 competed efficiently with each other for protein binding. Whereas oligonucleotide 2 only partially abolished the formation of oligonucleotide 1- and 3-protein complexes, oligonucleotides 1 and 3 abolished the formation of oligonucleotide 2-protein complex. In addition, the observation that oligonucleotide 1-, 2-, and 3-protein complexes, respectively, migrated with similar electrophoretic mobility suggests that the three oligonucleotides bind the same *trans*-acting factor.

Role of AP-1 Binding Sites and Identity of the Involved trans-Acting Factor—The −0.804 to −0.675-kb α1(I) procollagen promoter sequence contains 5 AP-1 binding sites which are preserved in the synthesized oligonucleotides and are homologous to the consensus AP-1 sequence (Fig. 5). Thus, the role of the AP-1 binding sites contained within the oligonucleotides was examined by competition experiments with excess unlabeled AP-1 consensus oligonucleotide (Fig. 6) and by using a consensus AP-1 oligonucleotide as a probe in mobility shift experiments as described by Kahari *et al.* (1992). The consensus AP-1 oligonucleotide abolished the formation of oligonucleotide 1-, 2-, and 3-protein complexes (Fig. 6). Mobility shift experiments with the labeled consensus AP-1 oligonucleotide showed the presence of DNA-protein complexes with nuclear extracts from normal and TSK fibroblasts (*bracket* in Fig. 7). Furthermore, it was found that markedly lower amounts of DNA-protein complexes were formed when nuclear extracts from TSK mice myocardial fibroblasts were examined, in comparison with nuclear extracts from normal cells (Fig. 7 and Table IV). These complexes were specific because they were competed out by excess unlabeled AP-1 oligonucleotide, but they were not affected by addition of salmon sperm DNA. Competition experiments showed that formation of these complexes was largely abolished by oligonucleotides 1, 2, or 3 (Fig. 7). As a control to determine the specificity of the findings of lower DNA binding with the AP consensus oligonucleotide in nuclear extracts from TSK fibroblasts compared with normal cells, we examined

DNA binding activity to the consensus SP-1 sequence (Fig. 8). We found that nuclear extracts from normal and TSK mice myocardial fibroblasts showed no differences in the amounts of DNA-protein complexes formed with the consensus SP-1 sequence (*arrows* in Fig. 8). These observations indicate that the nuclear extracts from TSK mice myocardial fibroblast strains have lower AP-1 sequence binding activity than normal fibroblasts.

In order to analyze the involvement of AP-1 *trans*-acting factor, supershift experiments with an AP-1 antibody were performed. Incubation of nuclear extracts with a polyclonal AP-1 antibody prior to the DNA-protein binding reaction resulted in supershifting of some of the oligonucleotide protein complexes (Fig. 9). Thus, the α1(I) procollagen promoter sequence between −0.804 and −0.675 kb binds AP-1 transcription factor. Collectively, since TSK nuclear extracts showed lower binding activity to a consensus AP-1 sequence as well as to AP-1 binding oligonucleotides spanning the mapped regulatory sequence, it can be inferred that altered interaction of AP-1 factor with the AP-1 binding sites in this region of the α1(I) procollagen promoter is involved in the up-regulation of expression of the α1(I) procollagen gene in the TSK fibroblasts.

DISCUSSION

The TSK mouse is an animal model for scleroderma, and it can be considered a model for myocardial fibrosis. The present study was undertaken to delineate the mechanisms responsible for the increased collagen deposition and collagen gene expression in the TSK mouse myocardium. Examination of the cellular origin of the myocardial collagen network showed that, whereas cardiomyocytes participate in the synthesis of type IV collagen, fibroblasts are the cellular origin of collagen types I and III (Eghbali *et al.,* 1988). Since fibroblasts are responsible for myocardial collagen synthesis, normal and TSK mice cultures of myocardial fibroblasts were established to examine the
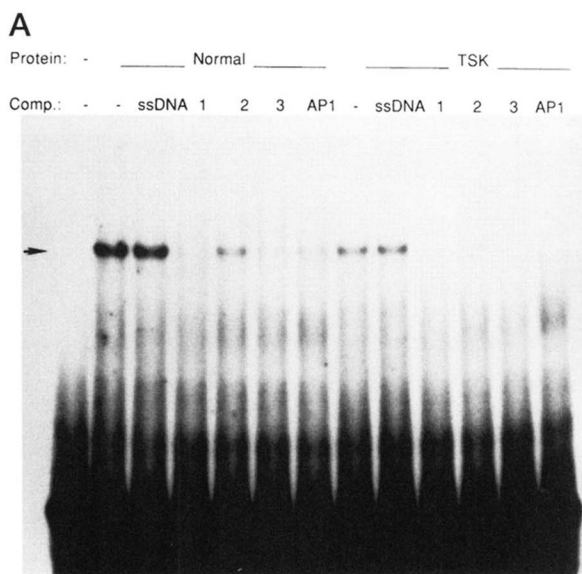
A

Protein: - _____ Normal _____ _____ TSK _____

Comp.: - - ssDNA 1 2 3 AP1 - ssDNA 1 2 3 AP1

B

Protein: - _____ Normal _____ _____ TSK _____

Comp.: - - ssDNA 1 2 3 AP1 - ssDNA 1 2 3

C

Protein: - _____ Normal _____ _____ TSK _____

Comp.: - - ssDNA 1 2 3 AP1 - ssDNA 1 2 3 AP1

TABLE IV

Comparison of DNA binding activities of nuclear extracts from normal (N) and TSK mice myocardial fibroblast strains 2, 3, and 4 for oligonucleotides 1, 2, 3, or AP-1

Fibroblast strain	Relative binding activity (N/TSK)			
	Oligo 1	Oligo 2	Oligo 3	Oligo AP-1
2	1.9	1.4	2.3	1.4
3	3.8	3.4	1.8	4.7
4	3.1	6.4	4.4	6.3
Average	3.0	3.7	3.0	4.1

Autoradiograms from mobility shift assays shown in Figs. 5, 6, and 7 were densitometrically scanned. The values obtained for DNA-protein complexes formed with oligonucleotides (oligo) 1, 2, 3, or AP-1 and nuclear extracts from each normal and TSK mice myocardial fibroblast strain are represented as the ratio of normal/TSK.
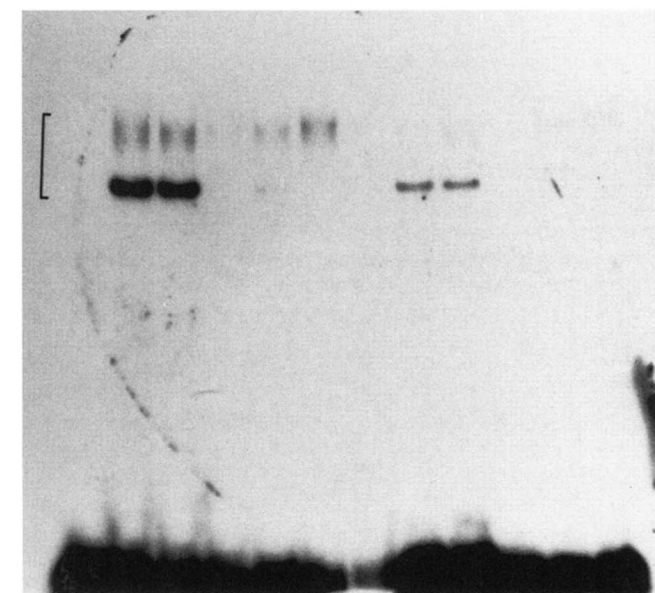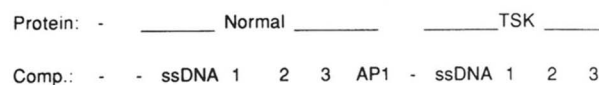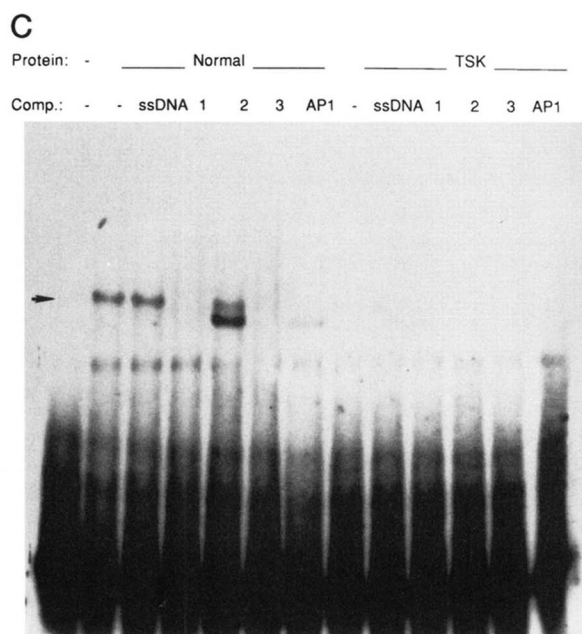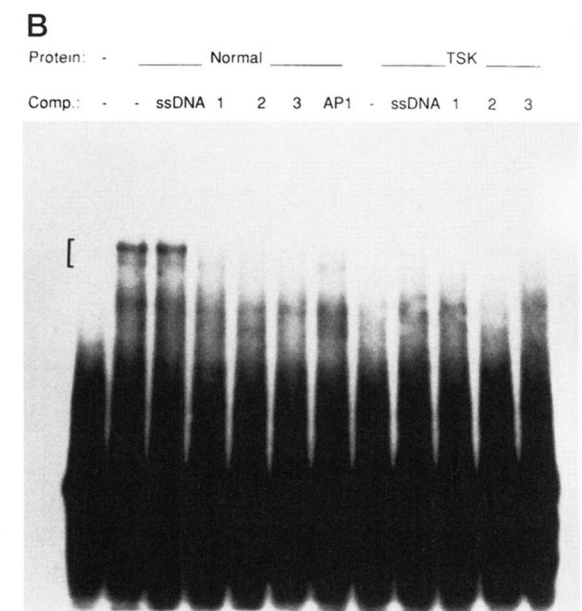
Protein: - _____ Normal _____ _____ TSK _____

Comp.: - - ssDNA 1 2 3 AP1 - ssDNA 1 2 3

FIG. 7. **Mobility shift assays with nuclear extracts from a normal and a TSK mice myocardial fibroblast strain and end-labeled double-stranded oligonucleotide AP-1.** The oligonucleotide AP-1-protein complexes (*bracket*) formed with 10 μg each of nuclear extracts from a normal and a TSK mice fibroblast strain incubated with 0.5 ng each of labeled oligonucleotide AP-1 are shown. The specificity of the oligonucleotide AP-1-protein complex was determined by inclusion of 100-fold molar excess of salmon sperm DNA and unlabeled oligonucleotide AP-1, respectively, in the binding reaction. Competition experiments (*Comp.*) were also performed with 100 fold molar excess of unlabeled oligonucleotides 1, 2, or 3.

mechanisms responsible for excessive collagen accumulation in the TSK myocardium.

All TSK mice myocardial fibroblast strains established showed increased synthesis of collagen, in comparison with normal (Table I). The observed increase in collagen biosynthesis by TSK mice myocardial fibroblasts was substantially higher than the 19% increase in hydroxyproline content seen in

FIG. 6. **Mobility shift assays with nuclear extracts from a normal and a TSK mice myocardial fibroblast strain and end-labeled double-stranded oligonucleotides.** The oligonucleotide-protein complexes (*arrow* or *bracket*) formed with 10 μg each of nuclear extracts from a normal and a TSK mice fibroblast strain incubated with 0.5 ng each of labeled oligonucleotide are shown. *A*, oligonucleotide 1; *B*, oligonucleotide 2; *C*, oligonucleotide 3. Competition experiments (*Comp.*) were performed with 100-fold molar excess of unlabeled oligonucleotides 1, 2, 3, or AP-1.
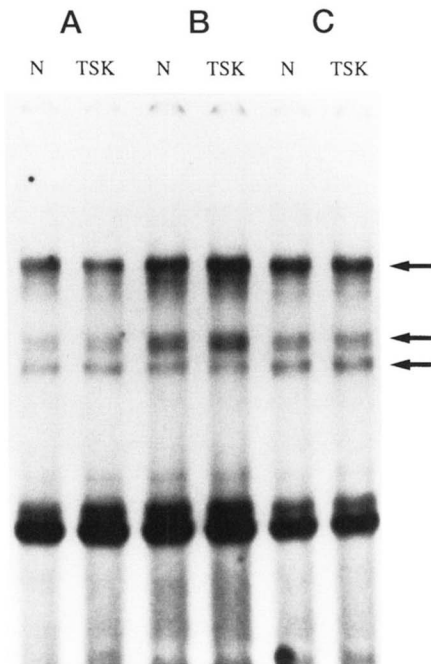
A   B   C

N TSK   N TSK   N TSK

FIG. 8. **Mobility shift assays with nuclear extracts from normal (N) and TSK mice myocardial fibroblast strains and end-labeled double-stranded oligonucleotide SP-1.** The oligonucleotide SP-1-protein complexes (*arrows*) formed with 10 μg each of nuclear extracts from normal and TSK mice fibroblasts incubated with 0.5 ng of labeled oligonucleotide SP-1 are shown: A, TSK strain 2; B, TSK strain 3; C, TSK strain 4.

1-year-old TSK mouse hearts, in comparison with normal (Osborn *et al.*, 1987) and higher than the 2-fold increase in [^{14}C]proline incorporation and collagen synthesis observed in myocardial organ cultures of TSK mice, in comparison with normal (Bashey *et al.*, 1993). Some explanations for the differences between *in vivo* and *in vitro* results include suppression of fibroblast biosynthetic activity *in vivo* by factors secreted by other cell types such as cardiac myocytes or due to the presence of a heterogeneous group of fibroblasts *in vivo* and the selection of fibroblasts with an abnormal phenotype during culture *in vitro*. The marked increase in cell layer-associated [^{14}C]collagenase-sensitive proteins in all TSK mice myocardial fibroblast cultures implies the formation of a well organized pericellular matrix. These observations indicate that the TSK mice myocardial fibroblast strains are different from TSK mice dermal fibroblasts, which in comparison with normal, show increases in ^{14}C-labeled proteins and collagen content only in media (Jimenez *et al.*, 1986). Interestingly, the cell layers of TSK mice myocardial fibroblast cultures contained mostly newly synthesized procollagen (Fig. 1) which is in contrast to cultured fibroblast cell layers from other tissues and species which mainly contain processed collagenous polypeptides (Bashey *et al.*, 1983, 1992). The above observations collectively suggest that the increased collagen deposition in the TSK mouse heart is largely due to the increased collagen biosynthetic activity of TSK mice myocardial fibroblasts.

Increased collagen biosynthesis in TSK fibroblasts was associated with coordinate increases in mRNA steady state levels and transcription rates of α1(I) and α1(III) procollagen genes. The average steady state levels of α1(I) and α1(III) procollagens mRNAs were 4.4- and 3.6-fold higher, respectively, in TSK mice myocardial fibroblast strains, in comparison with normal (Table II). The observed levels are much higher than the 41 and 63% increases, respectively, in α1(I) and α1(III) procollagen mRNA levels in the TSK mouse myocardial tissue (Chapman and Eghbali, 1990) but similar to the 5-fold increase in α1(I)

and α1(III) procollagen mRNA levels in TSK mice dermal fibroblast cultures (Jimenez *et al.*, 1986). Nuclear run-on assays showed that the increased α1(I) and α1(III) procollagen mRNA levels in TSK mice myocardial fibroblast strains were largely due to increased transcription of the respective genes (Fig. 3).

Comparison of the transcriptional activity of various α1(I) procollagen promoter deletion constructs (5′ end points from −5.3 to −0.084 kb) in normal and TSK mice myocardial fibroblasts indicated that the sequence between −0.675 and −0.804 kb of the α1(I) procollagen gene is strongly inhibitory in normal mice fibroblasts but its function is altered in TSK mice myocardial fibroblasts, thus resulting in increased expression of the gene in TSK fibroblasts (Fig. 4). Additional evidence supporting the inhibitory role of this region of the gene was provided by the studies of Simkevich *et al.* (1992), who found that deletion of the human α1(I) procollagen promoter sequence from −0.609 to −0.804 kb caused high collagen expression in transient transfections of mesenchymal and non mesenchymal cells (Simkevich *et al.*, 1992). Furthermore, nuclear extracts from TSK fibroblasts showed reduced binding activity to oligonucleotides encompassing the inhibitory region (between −0.675 and −0.804 kb) of the α1(I) procollagen promoter, in comparison with normal (Fig. 6). Hence, it can be concluded that the increased transcriptional activity of the α1(I) procollagen promoter in TSK mice myocardial fibroblast strains is due to the decreased available amounts and/or lower DNA binding activity of a transcriptional inhibitor that normally interacts with the strong inhibitory sequence between −0.675 and −0.804 kb of the α1(I) procollagen gene to modulate transcription. We also observed that oligonucleotides containing AP-1 binding sites corresponding to this region of the gene showed lower DNA-protein complex formation with nuclear extracts from TSK mice myocardial fibroblast strains (Figs. 6 and 7), and supershift experiments with an AP-1 antibody demonstrated that these oligonucleotides bind AP-1 protein (Fig. 9). Overall, these observations imply that decreased binding of AP-1 transcription factor to a strongly negative regulatory sequence between −0.675 and −0.804 kb of the α1(I) procollagen promoter may be responsible for the increased transcriptional activity of α1(I) procollagen gene which in turn resulted in the enhanced procollagen mRNA levels and collagen biosynthesis in TSK mice myocardial fibroblast strains.

Since AP-1 is not a single protein but a complex whose components are products of the *fos* and *jun* gene families, the specific components involved in the altered collagen expression in TSK fibroblasts need to be delineated. Each of the AP-1 components have been shown to have different functions. Furthermore, AP-1 has been reported to act as a transactivator (Lee *et al.*, 1987; Angel *et al.*, 1987) as well as a transrepressor (Franza *et al.*, 1988; Takimoto *et al.*, 1989; Lian *et al.*, 1991) of various genes. c-Jun and Jun D play opposing roles in growth regulation (Pfarr *et al.*, 1994). Regarding collagen genes it has been shown that an AP-1 site in the first intron of the human α1(I) procollagen gene acts either positively or negatively in different collagen producing cell lines (Katai *et al.*, 1992). The results reported here provide evidence that the regulatory sequence between −0.675 and −0.804 kb of the human α1(I) procollagen gene, which contains multiple AP-1 binding sites, is inhibitory in normal mice myocardial fibroblasts and may mediate transrepression by binding to AP-1 protein. These observations are consistent with studies of the effects of thyroid hormone and phorbol myristate acetate on myocardial fibroblasts (Eghbali *et al.*, 1991; Yao and Eghbali, 1992) which showed that thyroid hormone down-regulates collagen synthesis, type I procollagen mRNA steady state levels, and α1(I) procollagen promoter activity as well as induces c-*fos* and c-*jun*

A   B

Protein   -   10μg   -   10μg
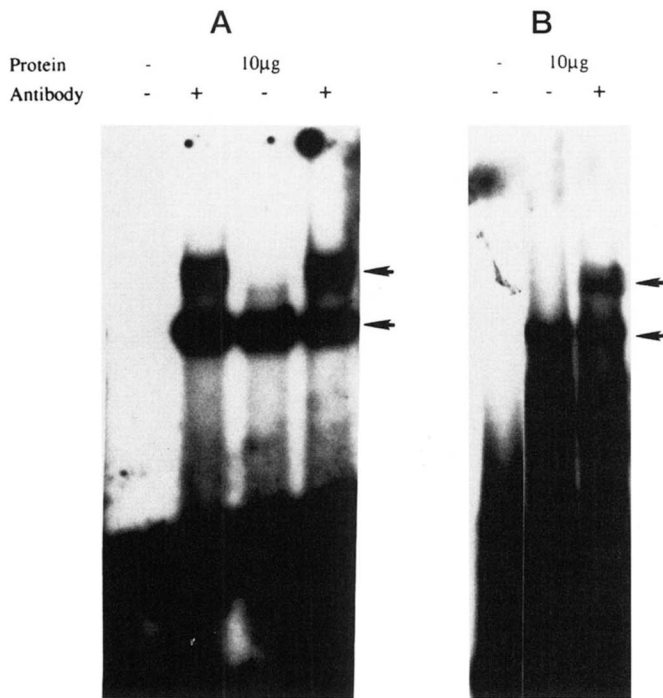Antibody   - +   -   +   -   -   +

FIG. 9. **Mobility shift assays of nuclear extracts from normal and TSK myocardial fibroblasts with end-labeled double-stranded oligonucleotides and a c-*jun*/AP-1 antibody.** Nuclear extracts from normal and TSK mice myocardial fibroblasts were incubated with AP-1 antibody (+) for 5 h at 4 °C prior to adding labeled oligonucleotides to the binding reactions for mobility shift assays. *A*, normal; *B*, TSK. Oligonucleotide 1 was employed in experiments shown in *A*, and oligonucleotide 3 was employed in experiments shown in *B*. Note supershifting of a large proportion of the oligonucleotides 1 and 3 in the presence of c-*jun*/AP-1 antibody.

protooncogenes in cultured myocardial fibroblasts (Yao and Eghbali, 1992). Similarly, treatment of cardiac fibroblasts with phorbol myristate acetate causes decreased collagen synthesis and procollagen mRNA steady state levels, effects which are associated with increased expression of protooncogenes c-*fos* and c-*jun* (Eghbali *et al.*, 1991).

The results reported here suggest that AP-1 is a transcriptional inhibitor of the expression of the α1(I) procollagen gene and that alterations in this regulatory mechanism are associated with the increased production of collagen in TSK mice myocardial fibroblasts. The link between the TSK mutation and associated multiple connective tissue abnormalities in the TSK mice is not yet known. However, it is unlikely that reduced AP-1 factor binding is a direct consequence of the TSK mutation, because given the pleiotropic effects of AP-1 factor, it would be expected that alterations in AP-1 factor expression/binding would result in a wider spectrum of abnormalities than those in connective tissue seen in the TSK mouse. It is possible

that the TSK mutation causes either activation or silencing of a gene product that leads to a cascade of events, one of which is inhibition of AP-1 expression or binding activity. However, cloning and identification of the TSK gene and characterization of its product(s), and identification of its functional role will be required in order to understand the cause of the observed collagen abnormalities in the TSK mouse.

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