Sp1 DNA Binding Efficiency Is Highly Reduced in Nuclear Extracts from Aged Rat Tissues*

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To explore the role of transcriptional factors in the genesis of the senescent phenotype, nuclear extracts from 4- and 30-month-old rat brains were analyzed for the presence of DNA-binding proteins able to interact with double-stranded oligonucleotides containing recognition sites for sequence-specific DNA-binding factors. Gel shift assays revealed that the DNA-binding efficiency of Sp1 is significantly reduced in aged animals compared to young ones, whereas CTF/NF1 and AP1 from young and old rat nuclear extracts bind their DNA targets with the same efficiency. The quantitative analysis of Sp1 by immunoblotting indicated that equivalent quantities and degrees of heterogeneity of Sp1 protein are present in both nuclear extracts, suggesting that the observed difference is not due to a different expression of this transcriptional factor. DNAse I footprinting of the heavy chain ferritin gene promoter, which contains a Sp1 binding site, demonstrated that the nuclear extract from 30-month-old rat brain does not protect the region involved in the regulation of the H ferritin gene by Sp1. This results in a reduction of about 50% of the expression of the H ferritin mRNA in aged rat brains. Furthermore, the Sp1 binding sites present in the SV40 early promoter are not protected in a DNAse I footprinting assay where a nuclear extract from 30-month-old rat brain was used as a source of DNA binding proteins. Liver nuclear extracts prepared from young and aged rats demonstrated that a decrease of Sp1 binding efficiency is similarly present in this tissue.

The rationale of genetic theories of aging is that the life-span of any species is determined by a gene expression program that controls all the aging steps and in turn the appearance of the senescent phenotype (1). Thus, aging could result from an intrinsic timing of gene regulation similar to that driving the molecular events of development (2, 3).

In addition to general observations indicating that the life-span and the timing of the appearance of the senescent phenotype are species-specific genetic characters (4, 5) and that aging is accompanied by an overall deregulation of gene expression (6, 7), evidence is accumulating that aging is governed by genetic mechanisms. In fact, the expression of many genes is altered in the aged animals; some of them are inducible genes such as those encoding T-kininogen, an acute phase protein (8), the heat shock protein hasp 70 (9, 10), the malic enzyme, and glucose-6-phosphate dehydrogenase (11). Other examples of changes in gene expression observed during aging concern the genes encoding the androgen-regulated senescence marker proteins (12), the X-linked ornithine carbamoyl transferase (13), and the neuronal calcium binding protein 28 kDa (14). Furthermore, many results support the hypothesis that the age-related decline in the functions of the immune system is a consequence of a decreased activity of several genes controlling these functions, such as those encoding interleukin-2 (15), interleukin-2 receptor (16), γ-interferon (17) and interleukin-3 (18). Lastly, differential screening of cDNA libraries allowed, in several cases, the identification of numerous mRNAs encoding unknown proteins, whose amount is different in young and in old tissues (19) or cultured cells (20).

Because the expression of a gene is often regulated at transcriptional level by the interaction between transcriptional factors and promoter elements, it is conceivable that, at least in some cases, the changes in gene expression occurring during aging could result from age-related modifications of one or more transcriptional factors. In these topics we are studying the age-related changes of the expression and of the activity of the most common transcriptional factors. Herein we report the observation that in senescent rat tissues the binding efficiency of the transcriptional factor Sp1 is highly reduced, although its gene appears to be normally expressed.

EXPERIMENTAL PROCEDURES

Animals—Wistar male rats were used in all the experiments. They were maintained under pathogen-free conditions and fed ad libitum. Aged rats were from our colonies of Wistar rats. These animals have a median life-span of 36 months.

Nuclear Extracts Preparation—Nuclei from young and aged rat brain were prepared as described by Gorsky et al. (21) with modifications. Briefly, minced tissue (15 g of rat brain corresponding to four to five organs) was brought up to 30 ml with homogenization buffer (10 mM Hepes, pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 2.4 mM sucrose, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml each of aprotinin, pepstatin, and leupeptin) and was homogenized using a motor-driven 30-ml Teflon-glass homogenizer. The homogenate was layered over 10 ml of cushion solution (2 mM sucrose, 10% glycerol, 0.15 mM spermine, 0.5 mM spermidine, and the mixture of protease inhibitors described above) and centrifuged at 24,000 rpm for 30 min at −2°C in an SW-27 rotor. The nuclear pellet was resuspended in 25 ml of a 93 (v/v) mixture of homogenization buffer and H2O, again using a Teflon-glass homogenizer. This nuclei suspension was again layered over the

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1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethyl)enemtilo]tetraacetic acid; ds, double-stranded.
The final concentration was 0.42 M NaCl. The tube was centrifuged at 31,000 rpm for 60 min in a Ti-75 rotor at 0 °C to pellet chromatin. 0.14 M NaCl, and the mixture of protease inhibitors and an equal tions. The pelletted nuclei were resuspended in 4 ml of 0.14 NEB (20 mM Hepes, pH 7.9, 25% glycerol, 0.25 mM EDTA, 0.125 mM EGTA, and the mixture of protease inhibitors) and an equal amounts of ammonium sulfate (0.3 g/ml) was added to the supernatant, and the precipitated proteins were sedimentated by a 30-min centrifugation at 32,000 rpm in a Ti-50.2 rotor (0 °C). The pellet was resuspended in dialysis buffer (20 mM Hepes, 0.25 % KCl, 20% glycerol, 0.25 mM EDTA, 0.125 mM EGTA, and the mixture of protease inhibitors) and dialyzed against 1 ml nuclear extract and dialyzed against 1 ml of buffer and 1 h against the same buffer. The protein concentration was determined according to Bradford (22).

Synthesis and Labeling of Oligonucleotides—Oligonucleotides were synthesized by the solid-phase phosphoramidite method (Applied Biosystem, 391 DNA Synthesizer). Oligo-GC is a ds 28-mer that contains three sites for the transcriptional factor Spl (23). Oligo-Ad-NF1 is a ds 28-mer utilized for the affinity purification of the transcriptional factor NF-1 from rat liver (24). Oligo-AP-1 is a ds 21-mer able to bind AP1 purified protein (25). The sequences of the three oligonucleotides are: oligo-GC 5′-ATCGGGCGGGCGGGG- GCGGGGCGGGGC-3′; oligo-Ad-NF1 5′-TTATTTTGGATTGAA- GCCAACATATGATAA-3′; oligo-AP-1 5′-CGCTTTGATGATCGACC- GCGGGGCGGGGC-3′. Other three oligonucleotides with a sequence complementary to the previous ones were synthesized. To generate double-stranded molecules, equal amounts of the two complementary single-stranded oligonucleotides were heated for 1 min at 90 °C and left to cool at room temperature. 1 pmol of ds oligos was incubated in the presence of 1 unit of T4 polynucleotide kinase (Promega). 1 µl of [γ-3²P]ATP (3,000 Ci/mmol, Amersham) in 20 µl of kinase buffer (50 mM Tris, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) at 37 °C for 30 min.

Gel Shift Assay and DNase I Footprinting—Gel retardation experiments were carried out by incubating a labeled double-stranded oligonucleotide (2 ng) with 4 µg of nuclear proteins in a buffer containing 20 mM Tris, pH 7.8, 8 % Ficoll, 50 mM KCl, 1 mM EDTA, 0.2 mM DTT, 3 µg poly(dI-dC) for 15 min at room temperature. When two oligonucleotides were used at the same time, 2 ng each of ds oligos were incubated with 4 µg of nuclear proteins.

DNase I footprinting assays were performed according to Lichtsteiner et al. (26) on the 160-bp fragment of the H ferritin promoter and on a 60-bp fragment of the SV40 early promoter. Briefly, 30 µg of nuclear extracts were incubated in 20 µl of 25 mM Hepes, pH 7.6, 5 mM MgCl₂, 34 mM KCl, 1 µg poly(dI-dC) and 3–5 ng of end-labeled probe, for 1 h on ice. The proteins were digested at 0 °C for 5 min with 2 µl of DNase I (Boehringer) 35 µg/ml. The reactions were stopped by the addition of 4 µl of 25 mM Tris-HCl, pH 8, 25 mM EDTA, 3 % sodium dodecyl sulfate, 5 µg of proteinase K, and 3 µg of tRNA at 65 °C for 20 min and precipitated with 2 volumes of ethanol. Samples were electrophoresed in a 7 M urea, 6% polyacrylamide gel.

Western Blot—Western blot was carried out according to Harlow and Lane (27). To control the quality and quantity of filter-bound proteins after electrotransfer, the nitrocellulose membrane (Schleicher & Schuell) was stained transiently with Ponceau S (Sigma). Rabbit antibody against Spl was used to detect the protein. Bound antibody was visualized by using peroxycade conjugated antibody to rabbit IgG (Sigma) and the ECL fluorescent system from Amersham.

Northern Blot—Total RNA from rat brain was purified by homogenizing tissues in 4 M guanidinium thiocyanate and collected by centrifugation over 5.7 M CsCl in an SW-50.1 rotor at 35,000 rpm for 20 h at 20 °C. RNA samples were electrophoresed through a 1.5 % formaldehyde agarose gel overnight at 25 V and then blotted onto a Nytran membrane. The probe was labeled with [α-3²P]dATP (3,000 Ci/mmol, Amersham) using a random priming kit (Pharmacia LKB Biotechnology Inc.). The filter was prehybridized in Church buffer (28) for 1 h and then hybridized overnight at 65 °C with heat-denatured double-stranded DNA probe (2 x 10⁶ cpm/ml).

RESULTS

To test the DNA binding activity of known transcriptional factors in aged and young rat tissues, we prepared nuclear extracts from 4- and 30-month-old rat brains as described under Experimental Procedures. 3²P-Labeled ds oligonucleotides, which contain recognition sites for sequence-specific DNA-binding proteins, were utilized as probes in gel shift assays. These experiments showed that oligo-GC (see Experimental Procedures) was shifted by Spl protein in the nuclear extract from young rat brain (Fig. 1, lane 3) whereas in the extract from senescent animals it was shifted with a very low efficiency (Fig. 1, lane 6). This phenomenon is peculiar of Spl factor, in fact, either CTF/NF1 (Fig. 1, lanes 4 and 7) or AP1 (data not shown) are able to shift the corresponding oligonucleotide with the same efficiency in young and aged nuclear extracts. To improve the accuracy of the evaluation of the different binding of Spl to oligo GC between young and aged nuclear extracts, the gel shift experiments were performed by mixing the oligonucleotides GC and Ad-NF1, recognizing Spl and CTF/NF1, respectively (Fig. 1, lanes 5 and 8). With this approach CTF/NF1 represents an internal control system for the normalization of the sample's processing and load. This allows us to calculate, by using a densitometric scanning on a longer exposure of the same experiment, that the amount of the oligo-GC shifted by Spl in aged rat extract is about 60-fold lower than that shifted in young rat extract.

To test whether the significant decrease of the Spl-shifted band was a consequence of a reduction of the amount of Spl in the aged tissue, Spl protein was measured by an immunoblotting with a polyclonal antibody. Fig. 2 shows that the quantity and the heterogeneity of Spl were similar in both nuclear extracts, indicating that the differences in DNA binding efficiency between young and senescent rat brain observed in gel shift experiments were not due to the presence of different levels of Spl in the two nuclear extracts.

To investigate if the lower efficiency of the binding of Spl protein in nuclear extract from aged rat brain has an effect on the genes on which Spl exerts its transcriptional activation, we performed a footprint analysis on the promoter of the human heavy chain ferritin with nuclear extracts from 4- and 30-month-old rat brain. It has been reported that the

FIG. 1. Detection of Spl and CTF/NF1 binding activity in nuclear extracts from young and aged rat brains. Lane 1, end-labeled oligo-GC; lane 2, end-labeled oligo-Ad-NF1; lane 3, oligo-GC preincubated with nuclear proteins from 4-month-old rat brains; lane 4, oligo-Ad-NF1 preincubated with nuclear extract from 4-month-old rat brains; lane 5, oligo-GC preincubated with nuclear proteins from 30-month-old rat brains; lane 7, oligo-Ad-NF1 preincubated with nuclear proteins from 30-month-old rat brains; lanes 5 and 8 show the Spl and CTF/NF1 shifts obtained by mixing equal amounts of oligos GC and Ad-NF1 and preincubating the mixture with nuclear extracts from 4- and 30-month-old rat brain, respectively. The arrow indicate the Spl-DNA and CTF/NF1-DNA complexes.
were detected with a polyclonal antibody against Spl. The 30-month aged rat brain.

17946 with 5 ng of end-labeled probe represented by the 160 bp upstream from the transcription start site of the H ferritin gene (29). Excess of unlabeled GC ds oligo; of nuclear proteins. The nuclear extract, preincubated with the probe in 9, protection using 15 and 60 pg of nuclear extracts, respectively. Protection of the transcription start site (29). Two protections can be distinguished in a DNase I footprint assay; the distal protection using 15 and 60 pg of nuclear extracts, respectively. The nuclear extract from young rat brain shows the two protections of the Spl binding regions (lane 3) spanning from 71 to 86 and from 89 to 98. The protections can be specifically competed with an unlabeled Spl double-stranded oligonucleotide (lane 4), whereas the same amount of a nonspecific oligonucleotide does not compete with the probe (lane 5). The nuclear extract from aged rat brain does not show the two protections (lane 6).

The differences in the Spl binding efficiency in the two transcriptional extracts suggested that ferritin gene expression could be altered in 30-month-old rat tissues. Fig. 5 shows the result of the Northern analysis of young and aged brain RNAs, hybridized with an H ferritin cDNA fragment. The experiment demonstrates a reduction of about 50% in the expression of the ferritin mRNA in the 30-month-old rat brain as calculated by laser densitometric scanning.

The phenomenon observed in brain extracts is not tissue-specific. In fact, liver nuclei were isolated from young and aged rats, and Spl present in liver nuclear extracts was examined for its ability to protect the A box of the H ferritin gene promoter (Fig. 6). This experiment demonstrates that, although the B box is similarly protected by young and aged liver extracts, Spl from 30-month-old rat liver extract exerts poor protective effect on the A box (Fig. 6, lane 3) compared to that of young liver (lane 2).

minimal promoter length required for the efficient transcription of the H ferritin gene is a fragment of about 160 bp from the transcription start site (29). Two protections can be distinguished in a DNase I footprint assay; the distal protection was demonstrated to bind Spl, and its deletion causes a significant decrease of the transcriptional efficiency of the promoter (29). Fig. 3 shows the DNase I footprint assay of the 160 bp of the H ferritin gene promoter; the fragment was incubated with the nuclear extracts from 4- and 30-month-old rat brains. The extract from young rat tissue showed the two protections previously reported (29) (lane 3): the A protection that spans from nucleotide -102 to -124 and the B region from -44 to -80 (lane 3). The A protection is not present when the reaction was carried out in the presence of two different unlabeled Spl double-stranded oligonucleotides, the GC ds oligo (lane 4) or the Sp-HSV ds oligo (lane 5) containing a well-characterized Spl binding site present in the promoter of the herpes simplex virus immediate early 3 (HSV IE-3) gene (30). On the contrary the same amount of a random oligonucleotide does not compete with the probe (lane 6). The extract from 30-month-old rat brain does not show the A protection (lane 7) even though the reaction was carried out with 60 pg of nuclear extract (lane 11), which is 2-fold higher than that used in all the other experiments. On the other hand, a footprint analysis performed with only 15 pg of nuclear extract from aged rat brain (lane 10) shows the same protection of the B box. The addition of Spl protein, purified from K562 cells, to nuclear extract from aged rat brain restores the A protection (lane 9).

To further demonstrate that the difference in binding efficiency that we observed in the two nuclear extracts was due to Spl, we performed the DNase I footprinting of a 60 bp fragment of the SV40 early promoter region (Fig. 4) which contains two Spl binding sites (31). The extract from young rat brain shows the two protections of the Spl binding regions (lane 3) spanning from 71 to 86 and from 89 to 98. The protections can be specifically competed with an unlabeled Spl double-stranded oligonucleotide (lane 4), whereas the same amount of a nonspecific oligonucleotide does not compete with the probe (lane 5). The nuclear extract from aged rat brain does not show the two protections (lane 6).

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transfer, the result of the rehybridization of the same filter with an oligo recognizing ferritin cDNA fragment (51). For the evaluation of the gel load and promoter with rat liver nuclear extracts.

We observed that: (i) oligonucleotides containing well demonstrated Spl binding sequences, are very poorly shifted by nuclear extracts from aged animals; (ii) the Spl binding sites present in the SV40 early promoter are not protected by these aged nuclear extracts; (iii) similarly, the Spl element present in the H ferritin gene promoter is not protected by the aged nuclear extracts. The reduced Spl binding efficiency is not a consequence of a decreased expression of the Spl gene. In fact, Western blot demonstrated the similarity of the amounts and of the electrophoretic migration pattern of Spl protein in young and aged animals. Furthermore, the presence of two identical bands (phosphorylated and not phosphorylated (32)) in young and old tissues, speaks against the existence of an alteration of the Spl protein due to an age-related mutation, also because it is very unlikely that different animals undergo the same mutational event. Therefore, the decrease of Spl binding efficiency to the GC box is probably caused by post-translational regulatory mechanisms. Three post-translational events have been demonstrated to affect Spl activity. The first concerns the O-linked glycosylation that enhances the ability of Spl to activate transcription (33). The second is the phosphorylation catalyzed by a DNA-dependent protein kinase (32) acting mainly on serine residues. The kinetic characteristics of this enzyme show that it is able to fully phosphorylate Spl in about 2 min only in the presence of the GC box. However, the binding efficiency of Spl to DNA does not seem to be affected by this phosphorylation, which, on the contrary, is most likely involved in the transactivation phenomenon (32). Accordingly, we carried out complementation experiments (data not shown) by mixing different ratios of young and old rat brain nuclear extracts, and the results do not show any increase in the binding efficiency of Spl of aged extract when mixed with the young brain extract. Similarly we did not observe any decrease of Spl binding efficiency of young extract when mixed with the aged nuclear extract.

The third regulatory event concerns the formation of Spl multimers either on single or on multiple GC elements (34). Similar to phosphorylation, the O-linked N-acetylglycosamine residues present on the Spl factor and the Spl multimerization seem to be very important for the transcriptional activation but not for the DNA binding.

The DNA binding of Spl is enhanced in the K562 leukemia cell line transfected with c-fes gene (35). The effect of p92c-fes on Spl binding activity is indirect, Spl being not phosphorylated on tyrosine (32). Similar to the observation in aged animals, in c-fes-transfected cells, the change in Spl binding to DNA is not related to a modification of the amount or of the electrophoretic mobility of the transcriptional factor. Therefore, the two phenomena could be governed by similar molecular mechanisms, although in our experiments a decrease of the p92c-fes tyrosine kinase cannot be invoked, because this proto-oncogene is mainly expressed in hematopoietic cells (36, 37) and absent or very poorly expressed in brain and liver.

Sp1 transcriptional factor, originally identified as the activator of Simian virus 40 (SV40) enhancer (38), is one of the most widely expressed transcription activating proteins. In fact, it is present in all mammalian cells examined so far (39), and numerous eukaryotic promoters contain the GC element (40–44) which interacts with Spl. The affinity of this binding significantly varies among the different promoters (45); in addition the amount of Spl varies up to 100 times among the diverse tissues examined (46). All these findings suggest that Spl is involved in the modulation of transcription of many eukaryotic genes. In these optics the present observation of a reduced DNA-binding efficiency of Spl in aged tissues could be proposed as one of the molecular mechanisms contributing to the altered gene expression in aging. Many experimental systems can be explored to evaluate this hypothesis: it is possible that many genes, whose expression is changed in aged animals (8–18) are directly or indirectly under the control of the transcriptional factor Spl. This could be the case of the impairment of the heat shock response observed in aging (9, 10) which is a consequence of a decreased expression of heat shock transcriptional factor(s) (47) and of the age-dependent decline of several functions of the immune system (15–18) whose molecular basis are still unknown.

It will be of interest to evaluate Spl binding efficiency in in vitro aged cultured cells, that represent an ideal experimental system in which the molecular basis of the described phenomenon could be evaluated. In these conditions it was
recently reported another dramatic change in the aged transcriptional machinery. This concerns with the repression of the c-fos promoter, by a possible dominant inhibitor, which prevents senescent fibroblasts to respond to serum (or to 12-O-tetradecanoylphorbole-13-acetate or cAMP) with the well described transient increase of c-fos mRNA levels (48). The possible relationship between this observation and our results are to be examined.

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