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Molecules and Cells

Identification of GATA2 and AP-1 Activator Elements within the Enhancer VNTR Occurring in Intron 5 of the Human *SIRT3* Gene

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Human *SIRT3* gene contains an intronic VNTR enhancer. A T > C transition occurring in the second repeat of each VNTR allele implies the presence/absence of a putative GATA binding motif. A partially overlapping AP-1 site, not affected by the transition, was also identified. Aims of the present study were: 1) to verify if GATA and AP-1 sites could bind GATA2 and c-Jun/c-Fos factors, respectively; 2) to investigate whether such sites modulate the enhancer activity of the *SIRT3*-VNTR alleles. DAPA assay proved that GATA2 and c-Jun/c-Fos factors are able to bind the corresponding sites. Moreover, co-transfection experiments showed that the over-expression of GATA2 and c-Jun/c-Fos factors boosts the VNTR enhancer activity in an allelic-specific way. Furthermore, we established that GATA2 and c-Jun/c-Fos act additively in modulating the *SIRT3*-VNTR enhancer function. Therefore, GATA2 and AP-1 are functional sites and the T > C transition of the second VNTR repeat affects their activity.

INTRODUCTION

SIRT3 is a NAD^+ dependent deacetylase which belongs to the evolutionarily conserved sirtuin family (Haigis and Guarente, 2006; Michan and Sinclair, 2007). SIRT3 protein has been localized to mitochondria (Cooper and Spelbrink, 2008; Lombard et al., 2007; Shi et al., 2005) but it has also been reported in the nucleus (Scher et al., 2007). In mice, during cold exposure, SIRT3 is induced in brown adipocytes where it promotes the expression of genes related to mitochondrial functions and thermogenesis (Shi et al., 2005). In addition, SIRT3 knockout mice exhibit a striking hyperacetylation of mitochondrial proteins, suggesting the existence of multiple mitochondrial substrates for SIRT3 (Lombard et al., 2007) including acetyl-CoA synthetase 2 (Hallows et al., 2006; North and Sinclair, 2007; Schwer et al., 2006), glutamate dehydrogenase and isocitrate dehydrogenase 2 (Schlicker et al., 2008). SIRT3 was shown to mediate the protective effect of NAD^+ on cell death induced by genotoxic stress (Yang et al., 2007). However, a pro-apoptotic role of SIRT3 has also been detected in pathways regulating cell survival (Allison and Milner, 2007).

Recently, SIRT3 has been implicated in the maintenance of basal ATP levels and the regulation of mitochondrial electron transport (Ahn et al., 2008).

By population studies we discovered two *SIRT3* polymorphisms, which were shown to be associated with longevity. First, by applying a genetic-demographic approach in a southern Italian population, we proved that a silent G477T polymorphism located in the third exon of the *SIRT3* gene is associated with survival of elderly males (Rose et al., 2003). Then, by carrying out molecular and functional studies, we discovered a Variable Number of Tandem Repeat (VNTR) located in intron 5 (Bellizzi et al., 2005). In particular, this VNTR was able to act as a transcription regulatory element *in vitro*, and the different alleles 1-6 (six alleles spanning from 1-6 repeats of a 72-bp repeat core) affected the expression of a reporter gene according to the number of VNTR repeats. In addition, a T > C transition located in the second repeat identified two VNTR allelic series (a and b) characterized by T or C, respectively, at the position 63 from the starting point of the second repeat. In particular, the allele 2b showed *in vitro* a reduction of the enhancer activity with respect to allele 2a. Interestingly, allele 2b was absent in males older than 90 years thus suggesting that this allele is detrimental to attain longevity. The T > C transition implied the presence or absence of a putative protein binding motif previously annotated as GATA-binding protein 3 (GATA3) site (Bellizzi et al., 2005). However, while the GATA3 factor is restricted to a minority of cell lines (Marine and Winoto, 1991), the GATA-binding protein 2 (GATA2) is expressed by a variety of cells, including the HeLa cells used in our study (Bellizzi et al., 2005; Dorfman et al., 1992). Therefore, we wished to verify if the GATA sequence was able to bind the GATA2 factor. On the other hand, both GATA2 and GATA3 are members of a family of transcription factors which bind the same consensus DNA sequence (Patient and MacGhee, 2002). The bioinformatics analysis also showed that GATA site overlapped partially with an Activator Protein-1 (AP-1) site recognized by the c-Jun/c-Fos heterodimeric complex (Chiu et al., 1988). For the sake of clarity, a schematic representation of the alleles 2a and 2b is given in Fig. 1. Aims of the present study were: 1) to check the

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binding of GATA2 and c-Jun/c-Fos factors to the putative binding sites; 2) to investigate the functional role of the two sites in the transcriptional regulation of the VNTR sequence, also by searching for a possible relationship between these sites and the different enhancer activity of the alleles 2a and 2b. As to point 1, we performed DNA Affinity Precipitation Assay (DAPA) in which we used GATA2 and c-Jun /c-Fos as DNA binding proteins. As to point 2, we carried out transient co-transfection experiments in HeLa cells by using luciferase reporter constructs containing the standard early simian virus 40 (SV40) promoter. Our results confirm the functionality of the GATA2 and AP-1 sites and show that the T > C transition is in fact responsible for the different enhancer activity of the alleles 2a and 2b.

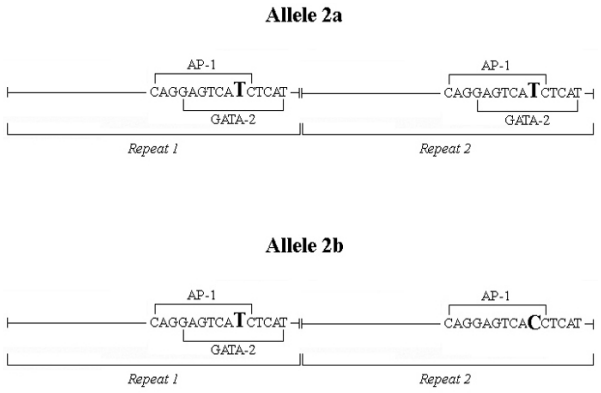

Fig. 1. Schematic representation of the alleles 2a and 2b. The T > C transition (bold letter) at the position 63 from the starting point of the second VNTR repeat (*Repeat 2*) and the putative GATA2 and AP-1 sites are shown.

MATERIALS AND METHODS

Plasmid constructs

pGL3-promoter vector and pRL-CMV vector were purchased by Promega; pGL3/allele2a and pGL3/allele2b reporter constructs, containing the allele 2a and the allele 2b, respectively, cloned into the SmaI site of the pGL3-promoter vector, were prepared as described in Bellizzi et al. (2005); expression constructs for both wild type GATA2 and GATA2 with C-terminal zinc finger deletion were previously described (Tong et al., 2000); c-Jun and c-Fos expression vectors were kindly provided by M. Maggiolini (Department of Pharmaco-Biology, University of Calabria, Italy). All plasmids were propagated in *Escherichia coli* Top10 strain.

Nuclear extract preparation

Nuclear extracts from HeLa cells were prepared starting from 10^7 cells (near confluent 10 cm plate). Cells were washed with phosphate-buffered saline (PBS, Invitrogen) twice. Then, cells were resuspended in 400 μl of ice-cold Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 nM $MgCl_2$) added just before use with 0.5 mM Dithiotreitol (DTT), 0.2 mM PhenylMethylSulfonylFluoride (PMSF) and 0.25 mM Leupeptin. The lysate was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 4°C at 14,000 rpm for 3 min. The supernatant was discarded, the pellet was resuspended by pipeting up and down in 50 μl of Buffer B (10 mM HEPES, pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 25% Glycerol, 1.5 nM $MgCl_2$) added just before use with DTT, PMSF and leupeptin as above and incubated in ice for 20 min. The sample was centrifuged at 4°C at 14,000 rpm for 2 min. Protein concentration was routinely measured by Bradford method as standard procedure. Extracts were divided in 15 μl aliquots, frozen in liquid nitrogen and stored at -80°C.

DNA affinity precipitation assay (DAPA)

Double-stranded oligonucleotides corresponding to alleles 2a and 2b were biotin end-labeled by using the LightShift Chemiluminiscent EMSA Kit (Pierce). Each labeling reaction was carried out in 50 μl of a mix containing 5 pmoles of oligo, 5X Reaction Buffer, 5 mM Biotin-N4-CTP, 10 U Terminal Deoxynucleotidyl Transferase (TdT, 2 U/μl). The reactions were incubated at 37°C for 1 h, stopped by adding 2.5 μl of 0.2 M EDTA pH 8.0 and then treated with 100 μl chloroform:isoamyl alcohol (24:1) to extract TdT. The 100 μg of nuclear extracts were mixed with 2 pmol of each biotinylated oligonucleotide in a final volume of 400 μl of binding buffer (10 mM HEPES, pH 7.9, 10% glycerol, 50 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$) and placed on ice for 45 min. Then 50 μl of streptavidin coated beads were added, and the samples were incubated for a further 3 h at 4°C with gentle agitation. The pellet (beads-proteins-oligonucleotides) was separated from the supernatant by centrifugation at 13,000 rpm for 5 min. After two washes with 1 ml of PBS, the bound proteins were released by boiling in Sodium Dodecyl Sulfate (SDS) loading buffer and then subjected to Western blot analysis. Proteins were separated by 10% SDS PAGE and transferred to nitrocellulose membrane. The membrane was blocked in 5% non fat milk in TBS-T (50 mmol/liter Tris-HCl, pH 7.5, 150 mmol/liter NaCl, 0.05% Tween 20) for 1 h at room temperature (r.t). GATA2 was detected with rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc., sc-9008). After incubation with primary antibody in TBS-T containing 1% milk for 2 h at r.t. or 16 h at 4°C, the membrane was washed extensively with TBS-T and then incubated with secondary anti-rabbit horseradish peroxidase-conjugated antibody (GE Healthcare) for 1 h at r.t.. After extensive washes with TBS-T, the membrane was visualized with ECL plus reagents (GE Healthcare). Then, the membrane was stripped for the detection with anti c-Jun and c-Fos rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc., sc-1694 and sc-52, respectively) to visualize the pull-down proteins.

Co-transfection experiments

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 4.5 g/L glucose and 2 mM L-Glutamine supplemented with 5% Fetal Bovine Serum (FBS, Invitrogen) and 1% Penicillin/Streptomycin (Invitrogen). The cells were cultured in a water-humidified incubator at 37°C in 5% CO_2 95% air. 1×10^5 HeLa cells were transferred into 24-well plates with 500 μl of regular growth medium/well the day before co-transfection.

Co-transfections were performed with the Fugene6 Reagent as recommended by the manufacturer (Roche Molecular Biochemicals) with a mixture containing: i) 1 μg of pGL3/allele2a and pGL3/allele2b constructs; ii) 1-6 μg of the expression vectors (GATA2 and c-Jun/c-Fos); iii) 2 ng of pRL-CMV (Promega), a plasmid that contains the *Renilla* luciferase gene under the cytomegalovirus promoter and which is utilized as an internal control to normalize the effects of transfection efficiencies. The cells were lysed 24 h after co-transfection, by applying 50 μl Passive Lysis Buffer of the Dual Luciferase Reporter Assay Kit (Promega) into each well of the 24-well plates. Twenty microliters of cell lysate were used for luciferase reporter assay with the same kit according to the manufacturer's protocol. Light intensity was quantified in a Lumat LB9507 luminometer (EG&

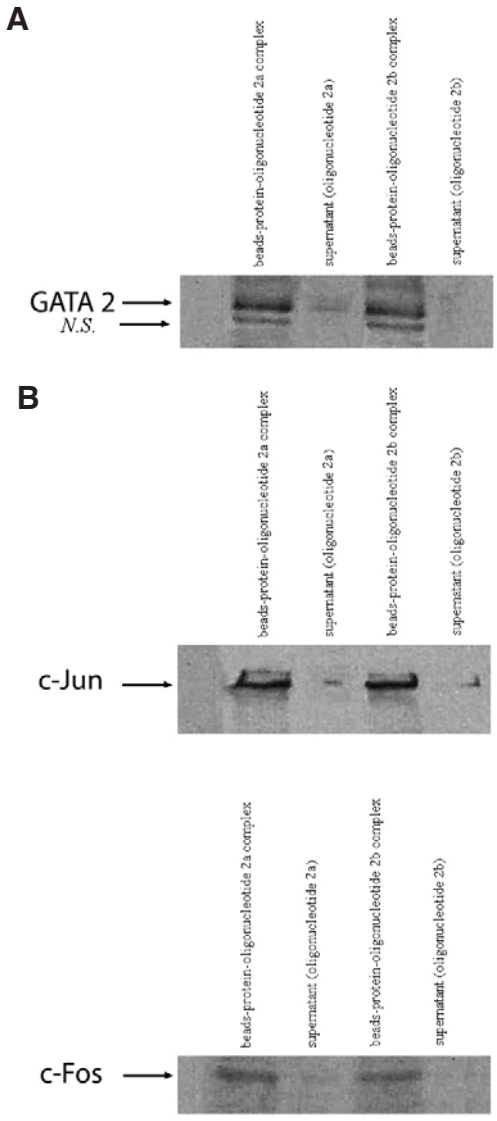

Fig. 2. DNA Affinity Precipitation Assay (DAPA) on the alleles 2a and 2b. The nuclear extracts were prepared and subjected to DNA affinity precipitation assay. 5′-biotinylated oligonucleotides corresponding to alleles 2a and 2b were used as probes. Proteins bound to the beads were eluted and subjected to Western blot analysis with antibodies anti-GATA2 (A) and anti c-Jun/c-Fos (B). Note that the N.S. band in (A) is a non-specific band due to GATA2-antibodies (sc-9008) as also reported by the customer (Santa Cruz Biotechnology, Inc.). Western blots of the supernatants separated by centrifugation from the beads-proteins-oligonucleotides 2a and 2b complexes are also shown.

G Berthold). The Luciferase activity of the reporter plasmids was normalized to the Renilla luciferase activity.

Statistical analysis

The Student's *t*-test for two independent samples was used to test the null hypothesis that the means of the luciferase activity are equal in the two samples analyzed (two-tailed test). The F test has been performed in order to decide to assume equal or unequal variances in the computation of the *t*-test. *T*-tests were considered significant when $p < 0.05$.

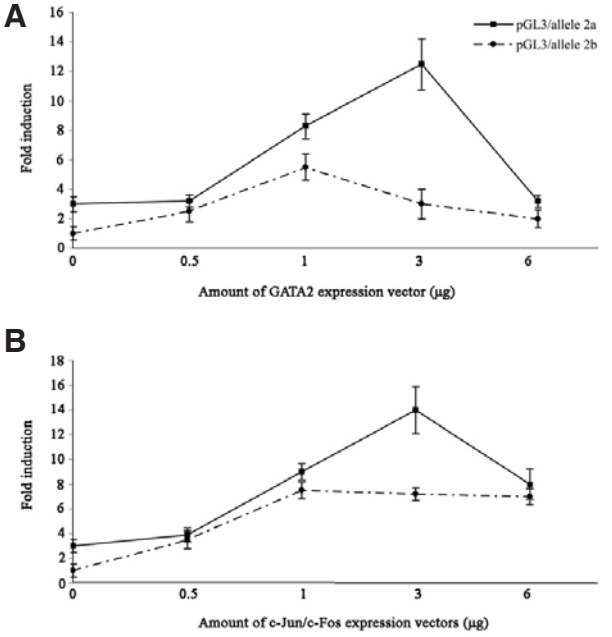

Fig. 3. Titration of GATA2 and c-Jun/c-Fos expression vectors. The pGL3/allele2a (solid line) and pGL3/allele2b (dashed line) reporter constructs were co-transfected with the indicated amounts (X-axis) of the GATA2 expression vector (A) or the c-Jun/c-Fos expression vectors (B). The luciferase activity of pGL3/allele2a and pGL3/allele2b reporter constructs is reported as fold induction with respect to the pGL3-Promoter vector (Y-axis). The fold induction values are the mean ± standard error mean of three independent duplicate experiments.

RESULTS

Binding of GATA2 and c-Jun/c-Fos to the alleles 2a and 2b

By using DAPA we tested whether GATA2 and c-Jun/c-Fos were able to bind the DNA sequence of the alleles 2a and 2b. As from Fig. 2, both GATA2 (A) and c-Jun/c-Fos (B) are able to bind 2a and 2b oligo probes, thus demonstrating that GATA2 and AP-1 sites are in fact present in both the alleles.

Relationship between GATA2 / AP-1 sites and enhancer activity of the alleles 2a and 2b

By co-transfections in HeLa cells we investigated the functional role of GATA2 and AP-1 sites in relationship to the enhancer activity of the alleles 2a and 2b.

As a first step, we performed a series of experiments aimed to determine the appropriate amounts of expression vectors to be used in co-transfections. To this purpose, we co-transfected 1 μg of pGL3/allele2a or pGL3/allele2b reporter constructs (Bellizzi et al., 2005) with increasing amounts of GATA2 and c-Jun/c-Fos expression vectors (Fig. 3). As for allele 2a, the maximum level of luciferase activity resulted when 3 μg of either GATA2 or c-Jun/c-Fos expression vectors were used. On the contrary, 1 μg of the expression vectors was sufficient to attain the maximum level of the luciferase activity in the case of allele 2b. Thus, in order to have the same experimental conditions for alleles 2a and 2b, we carried out the following co-transfections at the point of maximum of the function shown in Fig. 3 (3 μg and 1 μg of the expression vectors for alleles 2a and 2b, respectively).

Figure 4 shows the luciferase activity resulting from pGL3/al-

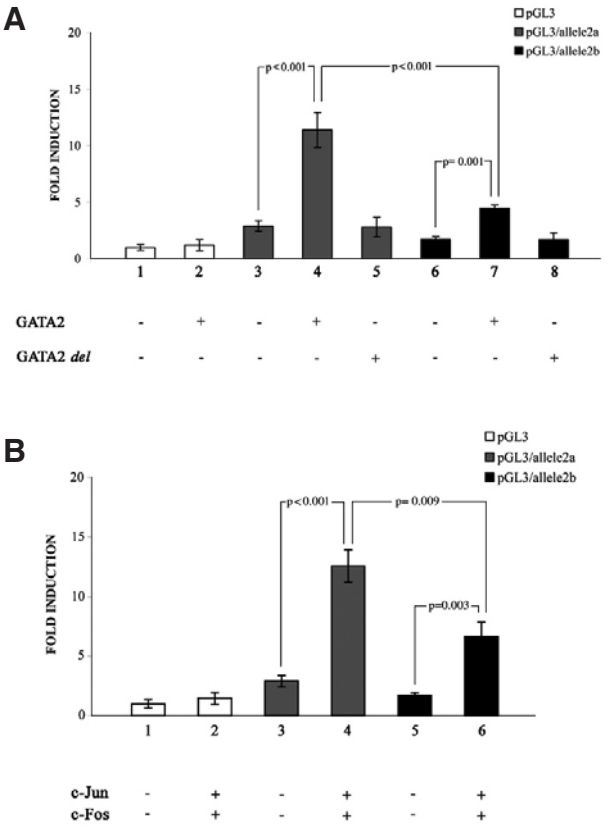

Fig. 4. Luciferase activity of the pGL3/allele2a and pGL3/allele2b reporter constructs in absence or in presence of GATA2 or c-Jun/c-Fos expression vectors. The pGL3/allele2a and pGL3/allele2b were co-transfected with GATA2 (A) and c-Jun/c-Fos (B) expression vectors. The luciferase activity of the two reporter constructs is reported as fold induction with respect to the pGL3-Promoter vector. The fold induction values are the mean ± standard error mean of three independent duplicate experiments. Controls: pGL3-Promoter vector (bar 1 in both (A) and (B)); pGL3-Promoter vector plus GATA2 (bar 2 in (A)) and c-Jun/c-Fos (bar 2 in (B)) expression vectors.

lele2a and pGL3/allele2b reporter constructs in the presence of GATA2 (A) or c-Jun/c-Fos (B) over-expression. In the same figure (Fig. 4A) we show the luciferase activity of both constructs in presence of GATA2 *del*, a GATA2 mutant with a deletion of the C-terminal zinc finger, which disrupts the GATA2 DNA binding. The over-expression of GATA2 increased the activity of both the reporter constructs (allele 2a: bar 3 *versus* bar 4; allele 2b: bar 6 *versus* bar 7). Therefore, GATA2 is able to increase the enhancer activity of both alleles 2a and 2b, but the GATA2 effect is influenced by the T > C transition (bar 4 *versus* bar 7). Co-transfections with GATA2 *del* expression vector confirmed the above results. In fact, luciferase expression returned to the basal value when GATA2 wild type was replaced by the mutated GATA2 (bars 5 and 8).

The over-expression of c-Jun/c-Fos factors significantly increased the activity of both the reporter constructs (allele 2a: bar 3 *versus* bar 4; allele 2b: bar 5 *versus* bar 6), although this effect is lower on allele 2b than allele 2a (compare bars 4 and 6 of Fig. 4B). What is more c-Jun/c-Fos had a stronger effect than GATA2 in modulating activity of the allele 2b [compare bar 7 (Fig. 4A) and bar 6 (Fig. 4B)].

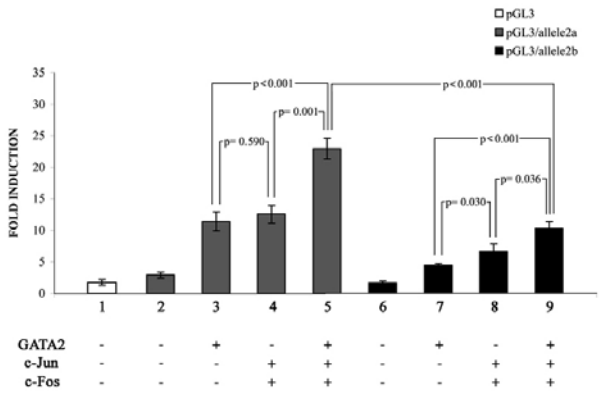

Fig. 5. Luciferase activity of the pGL3/allele2a or pGL3/allele2b reporter constructs in presence of both GATA2 and c-Jun/c-Fos expression vectors. The pGL3/allele2a and pGL3/allele2b were co-transfected with both GATA2 and c-Jun/c-Fos expression vectors. The luciferase activity the two reporter constructs is reported as fold induction with respect to the pGL3-Promoter vector. The fold induction values are the mean ± standard error mean of three independent duplicate experiments.

On the whole the results of Fig. 4 show that the enhancer activity of the *SIRT3*-VNTR repeat is positively modulated by GATA2 and c-Jun/c-Fos transcription factors. In addition the T > C transition leading to alleles 2a and 2b affects the effectiveness of the activity of the above factors.

Simultaneous presence of GATA2 and c-Jun/c-Fos expression vectors

To investigate the effect of the co-occurrence of GATA2 and c-Jun/c-Fos activators, we carried out co-transfection experiments by using the two reporter constructs together with the expression vectors encoding GATA2 and c-Jun/c-Fos. The results are shown in Fig. 5. We found different results as for allele 2a (bars 3-5) and allele 2b (bars 7-9). In particular, when two GATA2 sites and two AP-1 sites are present (allele 2a, see Fig. 1) no difference was observed in the activity of the reporter construct when GATA2 or c-Jun/c-Fos were over-expressed alone (bar 3 *versus* bar 4). On the contrary, when one GATA2 site but two AP-1 sites are present (allele 2b, see Fig. 1) the over-expression of c-Jun/c-fos induced a luciferase activity higher than the over-expression of GATA2 (bar 7 *versus* bar 8). Interestingly, in both 2a and 2b reporter constructs, an additive effect sustained by GATA2 and c-Jun/c-Fos on the activity of the reporter gene was observed (allele 2a: bar 5 *versus* bars 3 and 4; allele 2b: bar 9 *versus* bars 7 and 8). Moreover, the additive effect is affected by the T > C transition (bar 5 *versus* bar 9).

DISCUSSION

The present study was prompted by the discovery of a VNTR enhancer of the *SIRT3* gene; each VNTR allele was characterized by two allelic series, alleles a and b, different for a T > C transition occurring in the second repeat (Bellizzi et al., 2005). The intriguing observation was that the enhancer activity increased according to the repeat number independently of the a/b categorization, with a sole exception: one allele, allele 2b, was completely absent in males older than 90 years and showed an enhancer activity lower than that of allele 2a. The T > C transition implied the presence or absence of a putative

GATA binding motif. Moreover, the GATA motif was partially overlapped with an AP-1 motif not affected by the T > C transition.

The experiments here reported show not only that the GATA site is able to bind the GATA2 factor (Fig. 2) but also that it can modulate the VNTR enhancer activity (Figs. 4A and 5). These findings point towards an important role of the GATA2 site in the VNTR enhancing function and may explain why, by screening a sample of 900 individuals about, we have not found any allele totally lacking of the GATA2 site: in fact, the allele having one repeat only (allele 1a) contained the GATA2 site (Bellizzi et al., 2005). The functional role of GATA2 reported here is very interesting as to the involvement of GATA2 in the growth and survival of hematopoietic cells and the inhibition of adipogenesis (Ohneda and Yamamoto, 2002; Tong et al., 2003; Tsai and Orkin, 1997). This may provide a clue for the association observed between longevity and SIRT3 variability (Bellizzi et al., 2005; Rose et al., 2003).

An AP-1 site is located in the same DNA region: it overlaps in part with GATA2 (Fig. 1) and is able to bind c-Jun/c-Fos factors (Fig. 2). Furthermore, AP-1 is a functional site (Figs. 4B and 5). GATA2 and AP-1 sites are promoter/enhancer elements that play essential roles in the regulation of many genes in mammalian cells (Dannenberg et al., 2005; Majewski and Ott, 2002; Shaulian and Karin, 2002; Verde et al., 2007; Zhou et al., 2005). In addition, GATA2 and AP-1 sites are found closely associated in a number of genes, where they may interact to regulate the transcription process (Kawana et al., 1995; Masuda et al., 2004; Perkins and Davies, 2003; Yamashita et al., 2001;). Figure 5 confirms that also in the VNTR under study GATA2 and c-Jun/c-Fos factors cooperate in modulating the enhancer activity of the VNTR. Figure 5 shows also the important effect played by the T > C transition on the 2a/2b enhancer activity. In fact the panel 3-5 (allele 2a) clearly differs from the panel 7-9 (allele 2b): taking into account that the T > C transition affects the sequence of the GATA2 site but not that of the AP-1 site, the difference is not unexpected. Indeed, the activity of the allele 2a (two GATA2 sites and two AP-1 sites) is equally sustained by GATA2 and c-Jun/c-Fos factors. By contrast, the activity of the allele 2b is affected by c-Jun/c-Fos more than by GATA2, as expected considering that this allele has two AP1 site and only one GATA2 site. However, it is interesting to notice that the effect of c-Jun/c-Fos on the allele 2b is lower than that observed on the allele 2a. This observation suggests that the activity of c-Jun/c-Fos is affected by the absence of one GATA2 site in the allele 2b likely because of the overlapping of AP-1 site with the GATA2 site.

On the whole, our experiments show that GATA2 and AP-1 sites are functional to the enhancer activity of alleles 2a and 2b and that their functionality is affected by the T > C transition. We believe that the findings described above deserve further functional studies in order to investigate the effects of the alleles 2a and 2b *in vivo*, if their enhancer activity is tissue-specific or this activity is influenced by other genes.

GATA2 and AP-1 sites are largely present in the promoters of *SIRT1* (Voelter-Mahlknecht and Mahlknecht, 2006), *SIRT2* (Voelter-Mahlknecht et al., 2005), *SIRT3* (Bellizzi et al., 2007), *SIRT6* (Mahlknecht et al., 2006) and *SIRT7* (Voelter-Mahlknecht et al., 2006) genes. Interestingly, by alignment of the *SIRT3* genomic sequence with the other six genomic *SIRT* gene sequences, we found that GATA2 and AP-1 sites observed in the *SIRT3*-VNTR are conserved in *SIRT1* and *SIRT2* gene. It is noteworthy that *SIRT1*, *SIRT2* and *SIRT3* are the sirtuins showing highest level of similarity (Voelter-Mahlknecht and Mahlknecht, 2006). These observations seem to suggest that GATA2 and AP-1 sites could potentially modulate the complex network of sirtuin actions regulating complex phenotypes. In any case, the findings here reported may open a new avenue in exploring the interaction of these factors in the regulation of transcription.

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