

Functional Characterization of Promoter Variants of the Adiponectin Gene Complemented by Epidemiological Data

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OBJECTIVE—Adiponectin (*APM1*, *ACDC*) is an adipocyte-derived protein with downregulated expression in obesity and insulin-resistant states. Several potentially regulatory single nucleotide polymorphisms (SNPs) within the *APM1* gene promoter region have been associated with circulating adiponectin levels. None of them have been functionally characterized in adiponectin-expressing cells. Hence, we investigated three SNPs (rs16861194, rs17300539, and rs266729) for their influence on adiponectin promoter activity and their association with circulating adiponectin levels.

RESEARCH DESIGN AND METHODS—Basal and rosiglitazone-induced promoter activity of different SNP combinations (haplotypes) was analyzed in 3T3-L1 adipocytes using luciferase reporter gene assays and DNA binding studies comparing all possible *APM1* haplotypes. This functional approach was complemented with analysis of epidemiological population-based data of 1,692 participants of the MONICA/KORA S123 cohort and 696 participants from the KORA S4 cohort for SNP and haplotype association with circulating adiponectin levels.

RESULTS—Major to minor allele replacements of the three SNPs revealed significant effects on promoter activity in luciferase assays. Particularly, a minor variant in rs16861194 resulted in reduced basal and rosiglitazone-induced promoter activity and hypoadiponectinemia in the epidemiological datasets. The haplotype with the minor allele in all three SNPs showed a complete loss of promoter activity, and no subject carried this haplotype in either of the epidemiological samples (combined *P* value for statistically significant difference from a random sample was 0.006).

CONCLUSIONS—Our results clearly demonstrate that promoter variants associated with hypoadiponectinemia in humans substantially affect adiponectin promoter activity in adipocytes.

Our combination of functional experiments with epidemiological data overcomes the drawback of each approach alone. *Diabetes* 58:984–991, 2009

Adipose tissue produces and releases a variety of factors, which may be directly involved in the pathophysiology of obesity-associated insulin resistance (1). One of the most interesting candidates with respect to the development of metabolic syndrome and type 2 diabetes is the *APM1* gene that encodes the abundantly expressed protein adiponectin. Circulating adiponectin concentrations are negatively associated with insulin resistance and atherosclerosis and are decreased in humans with type 2 diabetes, coronary artery disease, or obesity (2). Animal experiments showed that administration of adiponectin reduces blood glucose levels, improves insulin resistance, and directly ameliorates endothelial dysfunction (3–5). Furthermore, low adiponectin levels are associated with other components of the metabolic syndrome, such as hypertension and dyslipidemia (6).

APM1 maps to chromosome 3q27, a region known to be linked to type 2 diabetes and the metabolic syndrome (7). In view of the important role of circulating adiponectin in the pathogenesis of major metabolic disorders, several studies have addressed the correlation of *APM1* SNPs with adiponectin levels. They revealed a significant correlation between two SNPs, rs266729 and rs17300539, and adiponectin levels (8–11). In one of these studies, the functional activity of both SNPs for transcriptional regulation as promoter elements was analyzed by luciferase assay (11). However, these experiments were performed in COS7 cells that do not express adiponectin and hence do not represent an ideal cell system for this type of analysis. We performed transfection experiments using mutated promoter constructs in 3T3-L1 adipocytes expressing endogenous adiponectin and analyzed DNA binding activity of different haplotype combinations of three promoter SNPs. Two of the selected SNPs are known to be associated with adiponectin levels and the third one lies in close proximity. This prompted us to assume that all three may be located in a transcriptionally functional element that may be altered by one or all SNPs. The relevance of these SNP haplotypes for human adiponectin levels was investigated in 1,692 participants of the MONICA/KORA (Cooperative Health Research in the Region of Augsburg) S123 cohort as well as in 696 participants of the KORA S4 cohort.

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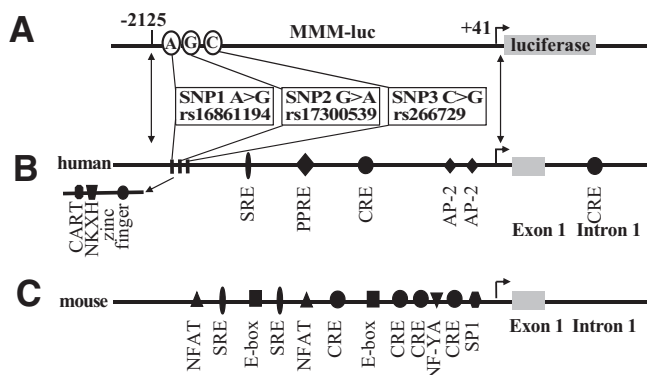


FIG. 1. Schematic overview of the used promoter constructs. **A:** Schematic overview of the luciferase reporter vectors used in this study for transfections. Genomic location of the here analyzed SNP1 (rs16861194), SNP2 (rs17300539), and SNP3 (rs266729) are marked (**B**). All experimentally verified transcription factor binding sites are shown for the human (**B**) and mouse locus (**C**), and the here analyzed SNPs are all located upstream of these sites. The genomatrix-predicted putative binding sites are depicted. SNP1 interferes with a putative CART binding site and SNP2 with a putative NKXH binding site (both sites for different families of homeobox proteins), and SNP3 interferes with a zinc-finger binding site.

RESEARCH DESIGN AND METHODS

SNP selection. We searched for SNPs in the promoter region of the *APM1* gene that 1) co-localize with putative transcription factor binding sites, 2) have been reported to be associated with adiponectin level or other adiponectin-related traits, and 3) lie in close genomic proximity. The first criterion is based on the assumption that SNPs may interfere with the functionality of a binding site, and the second should ensure previous epidemiological SNP association with adiponectin or related parameters. The rationale for the third criterion is the fact that transcription factor binding sites are often found in close proximity and build a functional module; the combination of different transcription factor binding sites is usually essential for regulation of transcription. If potential regulatory SNPs are found in such a potential module, it may enhance the probability that they are indeed functional SNPs. We hypothesized that SNPs combining both properties were most likely to alter a functional module in humans. The SNP2 (rs17300539, G>A) showed the strongest association with adiponectin levels in several studies (9–11). This SNP has been chosen together with two additional SNPs that both have shown association with type 2 diabetes (SNP1 = rs16861194 A>G; SNP3 = rs266729 C>G; in MONICA/KORA S123, rs1648707 A>C has served as proxy for SNP3 with linkage disequilibrium values of $r = 0.84$ and $D' = 1$). All three together are located in a small 80-bp part of the adiponectin promoter/enhancer region. Furthermore, all three SNPs lie within putative transcription factor binding sites that are shown in Fig. 1. These putative binding sites have been predicted using the Genomatix software (Genomatix, Munich, Germany).

Functional studies

Cell culture. The mouse preadipocyte cell line 3T3-L1 was cultured as described (12). To promote adipose differentiation, Dulbecco's modified Eagle's medium containing 10% fetal calf serum was supplemented with 250 nmol/l dexamethasone and 0.5 mmol/l isobutyl-methylxanthine for the first 3 days and 66 nmol/l insulin throughout the whole differentiation period.

Transfection of cells. 3T3-L1 cells were transfected on day 0, 6, and 8 of differentiation, respectively, using the Lipofectamine 2000 transfection reagent (Invitrogen, Karlsruhe, Germany). A total of 2 μ g DNA and 2 μ l transfection reagent were mixed according to the manufacturer's instructions and added to the cells for 4 h. Then 24–48 h after transfection, luciferase activity was measured using the dual-luciferase reporter assay (Promega, Mannheim, Germany). In all transfections, 0.2 μ g ubiquitin-promoter renilla luciferase vector (12) was cotransfected to normalize for transfection efficiency.

Cloning and mutagenesis of adiponectin promoter luciferase vectors. A 2,100-bp adiponectin promoter (bases $-2,125$ to $+41$) was PCR amplified from genomic DNA using an iProof High-Fidelity PCR Kit (Bio-Rad, Germany) and cloned into the pGL3basic luciferase vector (Promega, Germany) as described recently (13), using the primers depicted in Table S1 in the supplemental material (found in an online-only appendix at <http://dx.doi.org/10.2337/db07-1646>). The haplotype configuration of the cloned promoter was determined by sequencing and was shown to carry the major allele (M) of the three SNPs described above (MMM-luc). Using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, Germany) and the primers listed in Table S1

(supplemental material), the variants of the three SNPs were introduced in all remaining seven possible combinations (for primers, see Table S2). All vectors were sequenced to confirm the correct SNP variation combination.

Electrophoretic mobility shift assay. Probes for electrophoretic mobility shift assay (EMSA) were amplified by PCR from the above-described luciferase vectors carrying the eight different SNP combinations using the primers EMSA 5' and 3' (Table S1). The resulting 80-bp probe spans the APM1 gene corresponding to chromosome 3 position 188042104–188042183 (for sequence, see Figure S1 in the supplemental material). Primers contained a synthetic *HindIII* site, and resulting probes were cut to enable radioactive Klenow fill-in. EMSA was performed with 2–4 μ g nuclear protein extract and with 30,000–50,000 cpm of a 32 P-labeled probe as described previously (14). **Statistical analysis of transfection studies.** Overall, statically comparisons were performed using the Kruskal-Wallis test followed by pair-wise testing using the Dunn's multiple comparison test.

Epidemiological investigation

The KORA S4 and the MONICA/KORA S123 sample. The KORA Survey S4 (formerly known as S2000) is a population-based study of adults recruited from 1999 to 2001 conducted under the same conditions as the previous three surveys (S1, S2, S3) with patients recruited during the years 1984–1995 in the World Health Organization MONICA project. Details of the surveys are reported elsewhere (15). Study participants from all four surveys were from the study region of Augsburg (German nationality). Measures of weight and height were available to compute the BMI. All participants gave their written informed consent.

A subsample of the KORA S4 survey including 696 subjects aged 55–74 years with ~50% men was designed to address objectives regarding pre-diabetic stages. Adiponectin was measured in these subjects using the human adiponectin radioimmunoassay from Linco Research (St. Charles, MO) as described previously (16,17).

From the above-stated MONICA/KORA surveys S1, S2, and S3, a number of the 1,692 subjects aged 35–74 years with equal gender distribution were selected randomly from each survey as a subcohort sample (MONICA/KORA S123 sample) (18). Adiponectin levels were measured using the human adiponectin enzyme-linked immunosorbent assay from Merckodia (Uppsala, Sweden). The intra- and interassay coefficients of variation of control sera were 3.2% and 5.8%, respectively.

Genotyping. PCR primers were designed by Sequenom's MassARRAY Assay Design program. Genotyping analyses were carried out by means of matrix-assisted laser desorption ionization–time of flight analysis of allele-dependent primer extension products as described elsewhere (19). Genotyping calls were made in real time with MassARRAY RT software (Sequenom, San Diego, CA). Negative controls were included in all assays. In the 12.5% of randomly selected samples genotyped in duplicate, the discordance rate was 0.3%.

Statistical analysis of epidemiological data. Statistical SNP and haplotype association analysis was performed using the SAS procedure SURVEYREG to account for the sampling scheme in the MONICA/KORA S123 sample in the estimation of the standard errors of association estimates; in the KORA S4 sample, linear regression was applied using the GLM procedure. The logarithm of adiponectin was used as the outcome variable to yield a normal distribution. All analyses were adjusted for age, sex, and BMI. An additive as well as a dominant genetic model was applied. The minor allele frequencies of SNPs were computed, and linkage disequilibrium was assessed. SNPs were tested for Hardy-Weinberg equilibrium.

Haplotypes were estimated from genotypes via the expectation-maximization algorithm using the *R* statistics package (haplo.em) or SAS version 9.1 (20). Haplotypes were used in the regression models including all haplotypes except the most common haplotype to compute the association with adiponectin per copy of a haplotype adjusted for the other haplotypes compared with the group of subjects with two copies of the most common haplotype.

RESULTS

SNP variants and haplotypes in the two epidemiological KORA samples. In the two epidemiological cohorts KORA S4 and MONICA/KORA S123, we analyzed the three SNPs that we had selected for our investigation as candidates for an adiponectin-regulating role because of their nearby location in the APM1 promoter, because of their possible interference to transcription factor binding sites, and because of previous reports about association with adiponectin or related phenotypes. The SNPs analyzed were rs16861194 (SNP1), rs17300539 (SNP2), and rs266729 (SNP3) in KORA S4 and rs1648707 in MONICA/KORA S123 as proxy for SNP3 (as described in RESEARCH DESIGN AND

TABLE 1

Characteristics of SNPs in the sample of the KORA S4 study ($n = 696$) and the MONICA/KORA S123 study ($n = 1,692$)

	rs number	Position*	Call rate† (S4/S123)	Hardy-Weinberg equilibrium P value‡ (S4/S123)	Minor allele frequency (S4/S123)
SNP1	rs16861194	-11426	0.966/0.988	0.494/0.865	0.059/0.083
SNP2	rs17300539	-11391	0.951/0.990	0.347/0.897	0.091/0.090
SNP3§	Rs266729	-11377	0.967/0.989	0.773/0.990	0.278/0.333

*Position relative to the first position of the starting codon ATG; †proportion of genotyped sample, which successfully yielded a genotype; ‡testing for violation of HWE (exact); §rs266729 for KORA S4, the proxy rs1648707 for MONICA/KORA S123.

METHODS). The r^2 values as a measure of linkage disequilibrium were 0.006 (0.009) and 0.024 (0.179) for SNP1 compared with SNP2 and SNP3, respectively, and 0.038 (0.049) for SNP2 compared with SNP3 in the KORA S4 sample (and the MONICA/KORA S123 sample); hence, they are not in linkage disequilibrium. We statistically reconstructed the haplotypes; of the theoretically possible eight haplotypes across the three SNPs, we observed five (MMM, MmM, MMm, mMM, and mMm, with M and m indicating the major or minor allele, respectively). SNP and haplotype characteristics are given in Tables 1 and 2, respectively, indicating a large consistency of allele frequencies in the two cohorts.

APM1 promoter activity during adipocyte differentiation. The MMM adiponectin promoter construct containing the major allele M in all three SNPs was transfected into 3T3-L1 preadipocyte cells (d0) and 3T3-L1 adipocytes 6 and 8 days after induction of differentiation (d6 and d8). We measured a significant induction of luciferase activity during adipocyte differentiation (threefold on day 6 and fivefold on day 8, respectively) demonstrating the functionality of the promoter (Fig. 2).

Next, we focused on the five haplotypes observed in the KORA samples (MMM, MmM, MMm, mMM, and mMm). A significant overall difference between promoter activities was observed (Fig. 2A), and each promoter construct revealed significant induction of luciferase activity upon differentiation compared with respective transfections in undifferentiated cells in pair-wise comparisons. On day 6 of differentiation, we found a tendency of 50% higher promoter activity of the MMM promoter compared with the mMM, MmM, and MMm promoters. On day 8 of differentiation, the most striking difference was observed between the MMM and the mMM promoter, with MMM showing a threefold higher activity than mMM ($P < 0.05$).

TABLE 2

Characteristics of haplotypes in the sample of the KORA S4 study ($n = 696$) and the MONICA/KORA S123 study ($n = 1,653^*$)

	SNP1	SNP2	SNP3†	Frequency (S4/S123)
MMM	A	G	C	0.571/0.577
mMM	G	G	C	0.059/0.0003
MmM	A	A	C	0.091/0.090
MMm	A	G	G	0.278/0.249
Mmm	G	A	G	0/0
Mmm	A	A	G	0/0
mMm	G	G	G	0/0.083
mmM	G	A	C	0/0

Haplotypes are given by stating m or M for each of the three SNPs in a row indicating whether the haplotype exhibits the minor (m) or the major (M) allele at the SNP location. *For complete data for all three SNPs. †Depicted is the genotype C>G of rs266729 measured in KORA S4; the proxy rs1648707 with genotype A>C (not depicted) was measured in MONICA/KORA S123.

Notably, both promoters with the minor allele at the SNP1 position (mMM and mMm) revealed impaired basal promoter activity compared with the MMM promoter already in preadipocytes.

Next, we transfected cells with promoters regulated by the theoretically possible, using nonexisting haplotypes in the epidemiological samples (Mmm, mmM, and mmm), and observed the strongest impact by the threefold major to minor allele alteration (mmm promoter) with a complete loss of basal promoter activity in preadipocytes. Additionally, the mmm promoter was almost resistant to transcriptional activation during differentiation supporting the importance of these sites for transcription of the *APM1* gene. Interestingly, all promoters with the minor allele at the SNP1 position showed the strongest reduction of basal promoter activity or altered kinetic of activity during differentiation compared with the MMM promoter, suggesting a crucial role of SNP1 for promoter activation.

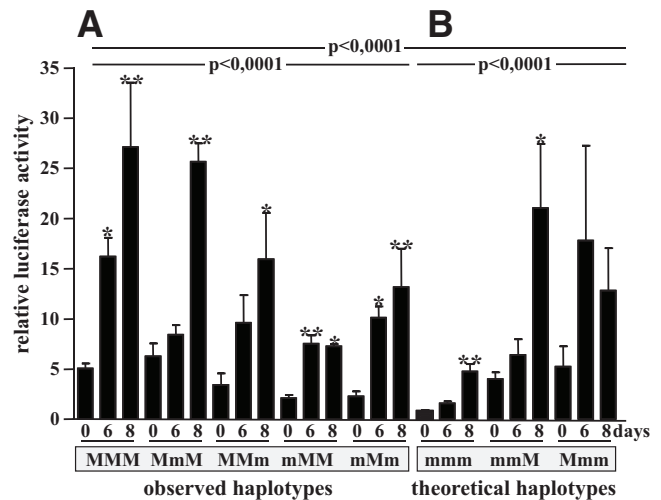


FIG. 2. *APM1* promoter activity during differentiation. Transient transfection of 3T3-L1 cells at different stages of adipogenic differentiation with the indicated *APM1* promoter constructs is shown. A total of 1 μ g of the indicated promoter construct (MMM = *APM1* promoter with the three described SNPs in the major configuration, m = minor variant, M = major variant) was transfected into 3T3-L1 cells at the indicated day of differentiation (day 0 = preadipocytes, day 6 and 8 = 6 or 8 days after induction of differentiation). A total of 0.1 μ g ubiquitin-renilla vector was cotransfected for normalization of the transfection. The haplotypes observed in KORA samples are depicted separately from the theoretically existing but not observed haplotypes. Cells were harvested 24 h after transfection. Results are shown as the ratio of firefly-/renilla-luciferase activity and the mean of minimal five independent experiments \pm SD. The Kruskal-Wallis overall comparison of all constructs and observed/theoretical haplotypes is indicated with P values; comparison of the day 0, 6, and 8 values for each construct were $P < 0.001$ for MMM, $P < 0.001$ for MmM, $P < 0.05$ for MMm, $P < 0.001$ for mMM, $P < 0.001$ for mMm, $P < 0.005$ for mmm, $P > 0.05$ for mmM, and $P < 0.01$ for Mmm. The significance of Dunn's multiple comparison test comparing the day 0 value for each construct with its values at day 6 and day 8, respectively, is indicated with asterisks: * $P < 0.05$ and ** $P < 0.01$.

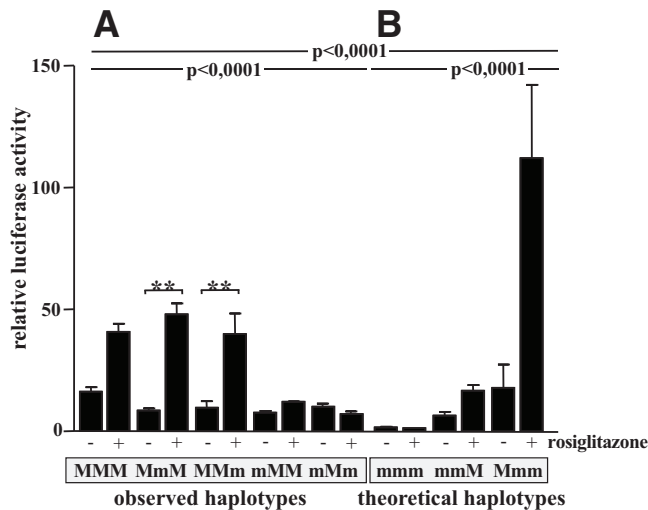


FIG. 3. Inducibility of different haplotypes by rosiglitazone. Transient transfection of 3T3-L1 cells with the indicated adiponectin promoter constructs at day 6 after induction of differentiation is shown. A total of 1 μ g of the indicated promoter construct was transfected into 3T3-L1 cells. A total of 0.1 μ g ubiquitin-renilla vector was cotransfected for normalization of the transfection. The haplotypes observed in MONICA/KORA S123 or S4 survey are separately depicted from the theoretical existing, but was not observed in patients. At 24 h after transfection, cells were induced with 1 μ mol/l rosiglitazone for 24 h as indicated. Cells were harvested 24 h after transfection. Results are shown as the ratio of firefly-/renilla-luciferase activity and the mean of minimal three independent experiments \pm SD. Kruskal-Wallis overall comparison of all constructs and of frequent/theoretical haplotypes is indicated with a P value, followed by the Dunn's multiple comparison test comparing the uninduced (-) with the respective rosiglitazone-induced (+) cells for each construct, as indicated with ** $P < 0.001$.

Impact of rosiglitazone on APM1 activation depending on SNP variant combinations. To investigate whether the different haplotype constructs had an impact on the inducibility of the *APM1* gene promoter, we transfected 3T3-L1 adipocytes on day 6 after induction of differentiation and determined luciferase activity in the presence or absence of rosiglitazone. Promoters with MMM, MmM, MMm, or Mmm haplotypes revealed a two- to fivefold induction of luciferase activity after treatment with rosiglitazone compared with control treated cells (Fig. 3). In contrast, all other haplotypes with the minor variant at the SNP1 position (mMM, mMm, and mmm) showed no response upon rosiglitazone treatment.

Influence of APM1 SNP variations on DNA binding activity. To analyze whether these haplotypes have an impact on DNA binding activity of nuclear proteins, we performed EMSAs using nuclear extracts from undifferentiated 3T3-L1 (preadipocytes) and in vitro differentiated 3T3-L1 adipocytes (day 6 after induction of differentiation) and DNA probes with all eight possible haplotypes. We found one major complex and some minor slower migrating complexes using the DNA probe with the major SNP variants (MMM) and nuclear extracts from preadipocytes. Nuclear extracts from differentiated adipocytes revealed a slight decrease of the major complex and increased binding of a slower migrating complex. Most haplotypes showed similar patterns of DNA binding compared with MMM. In contrast, the mMM probe revealed strongly reduced DNA binding activity with nuclear extracts from preadipocytes, but comparable binding of major and minor complexes in differentiated adipocytes. Finally, we performed EMSAs with nuclear extracts of preadipocytes and adipocyte cultures that were induced with rosiglitazone to investigate whether stimulation affects DNA binding activity.

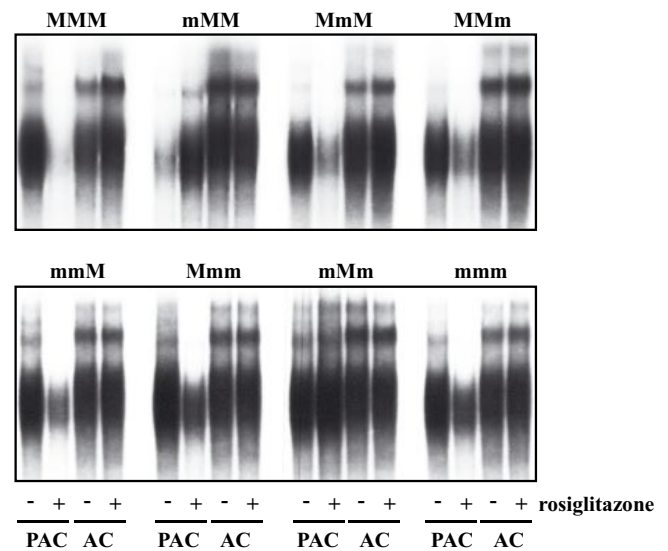


FIG. 4. DNA binding activity of different SNP variant combinations. An 80-bp fragment, described in RESEARCH DESIGN AND METHODS, was radioactively labeled, incubated with 2 μ g of the indicated protein extracts, and separated on a gel as described. AC, 3T3-L1 adipocyte; PAC, 3T3-L1 preadipocyte; cells were induced with 1 μ mol/l rosiglitazone (+) or with DMSO control (-).

Rosiglitazone treatment of preadipocytes abolished protein binding to DNA probes with MMM and the most other haplotypes (MmM, MMm, mmM, Mmm, and mmm), whereas no inhibition of DNA binding was found for the mMm variant. Moreover, we detected restored DNA binding activity for the mMM variant. Surprisingly, rosiglitazone treatment of differentiated adipocytes had no major impact on DNA binding activity (Fig. 4).

Association of SNPs and haplotypes with circulating adiponectin in the epidemiological samples. Tables 3 and 4 summarize the results of SNP and haplotype association analyses in the MONICA/KORA S123 (1,692 participants) and the KORA S4 (696 participants) samples. Subjects carrying the minor allele of SNP1 showed consistently lower circulating adiponectin levels in both cohorts, which was statistical significant in the larger MONICA/KORA S123 sample ($P = 0.001$), but not in the smaller KORA S4 sample. Consistent to the SNP1 finding, all haplotypes found in the studies containing the minor allele for SNP1 showed reduced adiponectin level, which was statistically significant for the more frequent mMm ($P = 0.009$). This observation of lower adiponectin level for the SNP1 minor allele and the respective haplotypes is in line with the promoter assay finding of a reduced activity for these haplotypes. Subjects carrying the minor allele in SNP2 showed a significant increase in adiponectin levels in both KORA samples ($P = 0.00005$ and $P < 10^{-9}$), which was in line with haplotype analysis for haplotype MmM ($P < 0.0001$ and $P = 0.0002$), but did not fit with the promoter activity assays. SNP3 showed decreased adiponectin levels in all studies, which was statistical significant in the larger MONICA/KORA S123 sample ($P = 0.00001$). Three haplotypes (mmM, Mmm, and mmm) were neither present in any subject of the KORA S4 nor in the MONICA/KORA S123 sample. Given the sample size of 1,676 (696) in the MONICA/KORA S123 (KORA S4) sample and the haplotype frequency of 0.0025 (0.0015) as expected from the minor allele frequencies of the three

TABLE 3

SNP association analysis in the KORA S4 sample ($n = 696$) and in the MONICA/KORA S123 sample ($n = 1,692$)

	SNP	Genotype	n	Mean* ($\mu\text{g/ml}$)	Coefficient (P)	
					Additive \dagger	Dominant \ddagger
S123 ($n = 1,692$)	SNP1	AA	1407	11.2062	Reference	Reference
		AG	253	10.5341	-0.0602 ($P = 0.0014$)	-0.0634 ($P = 0.002$)
		GG	12	10.1812		
	SNP2	GG	1388	10.7692	Reference	Reference
		AG	274	12.7478	0.1665 ($P < 10^{-9}$)	0.1748 ($P < 10^{-9}$)
		AA	13	14.6926		
	SNP3 \S	AA	745	11.4570	Reference	Reference
		CA	743	10.8979	-0.0502 ($P = 0.00001$)	-0.0598 ($P = 0.0001$)
		CC	185	10.3596		
S4 ($n = 696$)	SNP1	AA	596	8.929	Reference	Reference
		AG	73	8.536	-0.0362 ($P = 0.507$)	-0.0248 ($P = 0.6677$)
		GG	3	4.967		
	SNP2	GG	544	8.536	Reference	Reference
		AG	115	10.164	0.1897 ($P = 0.00005$)	0.2042 ($P = 0.00003$)
		AA	3	9.7		
	SNP3 \S	CC	349	9	Reference	Reference
		GC	274	8.876	-0.0287 ($P = 0.3273$)	-0.0185 ($P = 0.6167$)
		GG	50	8.422		

Data are from linear regression on log(adiponectin), adjusted for age, sex, and BMI and survey (for the S123 sample) using an additive or a dominant genetic model. *Geometric mean of adiponectin concentrations in micrograms adiponectin per milliliter serum. \dagger Mean change in log(adiponectin) per copy of the minor allele. \ddagger Mean change in log(adiponectin) for subjects to the indicated reference (e.g., SNP1 with the AG or GG compared with the AA). \S For SNP3 in the case of KORA S4, the genotype C>G of rs266729 is depicted; in the case of MONICA/KORA S123, the genotype A>C of the proxy rs1648707 is depicted.

SNPs, the finding of zero subjects with the mmm haplotype was statistically significantly different from what would have been expected by chance ($P = 0.0167$ in MONICA/KORA S123, $P = 0.3534$ in KORA S4, $P = 0.0059$ for both samples combined). This observation is in line with the observation of a complete loss of promoter activity.

DISCUSSION

Recent epidemiological studies support the concept that SNPs in the *APM1* gene are associated with type 2 diabetes and other metabolic disorders in several populations (6). In the current study, we investigated three different SNPs (SNP1 = rs16861194, SNP2 = rs17300539, and SNP3 =

rs266729 or rs1648707) in the *APM1* gene promoter region located within an 80-bp region of the promoter that are known for their association with circulating adiponectin levels or related phenotypes (9–11). We applied an approach of combining functional experiments with epidemiological data and showed that these SNPs influence basal and inducible *APM1* promoter activity in 3T3-L1 adipocytes accompanied by alterations in DNA binding activity. In human epidemiological studies, we presented SNP and haplotype association analyses of two population-based samples of the MONICA/KORA studies, which was consistent with most of our functional findings.

Intriguingly, the constructed promoter with the minor allele (mmm) in all SNPs was almost completely inactive

TABLE 4

Haplotype association analysis in the KORA S4 sample ($n = 696$) and the MONICA/KORA S123 sample ($n = 1,653^*$)

	Haplotype	n	Geometric mean \dagger	Coefficient	
S123 ($n = 1,676$)	MMM	0/1/2	300/799/554	11.343/11.035/11.029	Reference
	MmM	0/1/2	1,368/272/13	10.761/12.738/14.683	0.15641 ($P < 0.0001$)
	MMm	0/1/2	927/628/98	11.298/10.832/10.792	-0.022 ($P = 0.1009$)
	mMM	0/1/2	NA	NA	NA
	mMm	0/1/2	1389/252/12	11.203/10.520/10.174	-0.0489 ($P = 0.0091$)
S4 ($n = 696$)	MMM	0/1/2	119/356/221	9.003/8.878/8.758	Reference
	MmM	0/1/2	578/115/3	8.597/10.164/9.7	0.1775 ($P = 0.0002$)
	MMm	0/1/2	352/294/50	8.993/8.777/8.422	-0.0155 ($P = 0.603879$)
	mMM	0/1/2	620/73/3	8.918/8.536/4.967	-0.019 ($P = 0.733164$)
	mMm	0/1/2	NA	NA	NA

Results from linear regression models on log(adiponectin), adjusted for age, sex, and BMI, survey (for the S123 sample), and the other haplotypes, with MMM being the reference using an additive genetic model. Haplotypes are depicted by m and M for the minor or major allele, respectively, in SNP1, SNP2, and SNP3. 0/1/2 = number of reconstructed haplotype copies. *For complete data for all three SNPs. \dagger Geometric mean of adiponectin concentrations ($\mu\text{g/ml}$) per copy of the reconstructed haplotypes.

with regard to basal activity and differentiation- or rosiglitazone-induced activity. Our results clearly demonstrate the functional relevance of these SNPs for activation of the *APM1* promoter by interfering with transcription factor binding sites. Indeed, we found specific binding of nuclear proteins to a DNA probe containing all three minor alleles as well as changes in the pattern of DNA-protein complexes upon adipocyte differentiation and partly also upon rosiglitazone stimulation. Given the highly reduced promoter activity and low circulating adiponectin levels being associated with increased risk of severe diseases such as type 2 diabetes and coronary heart disease (3,4,21–27), one may speculate that the mmm haplotype affects adiponectin expression in vivo to an extent that might be disadvantageous. This hypothesis is supported by our epidemiological finding that none of the 2,340 subjects in our analysis carried this haplotype, which was highly significantly different from what would have been expected by chance ($P = 0.0059$). Yet, further studies in humans are necessary to support this hypothesis. The importance of haplotype combination has also been shown for SNP2 and SNP3, which in combination, increases the risk of diabetes (28).

The transcription factors involved in the regulation of the *APM1* promoter were analyzed by several groups (29–31); however, most studied promoter regions do not contain the SNPs analyzed here. One study demonstrated slightly higher promoter activity upon deletion of the promoter region containing these SNPs (13). However, such deletion of promoter regions removes all regulatory sites and hence does not allow a SNP-specific analysis concerning the influence on binding characteristics of transcriptional activators or repressors. Indeed, our EMSA experiments revealed the existence of specific DNA binding complexes that are affected by adipocyte differentiation and rosiglitazone stimulation. At least two minor alleles in the haplotype (mMM or mMm) exhibited obvious alterations in DNA binding complexes. Surprisingly, the presence of relevant DNA binding factors was already found in preadipocytes. It has to be considered that epigenetic mechanisms may also be involved in the regulation of the analyzed promoter. It is known that transcription of the *APM1* gene is regulated by histone acetylation (32). Interestingly, DNA binding activity upon rosiglitazone stimulation critically depends on the combination of several SNP variants. This supports the view that this promoter region represents a functional module with binding of various proteins that interact and build a more complex structure. As an example, we found alterations in DNA binding activity using the DNA probe with the minor SNP1 allele and nuclear extracts from preadipocytes. Further introduction of a minor allele in SNP2 restored normal DNA binding, whereas introduction of a minor allele in SNP3 resulted in a different pattern of DNA binding activity.

A potential limitation of our study is that mouse 3T3L1 cells may differ from human adipocytes regarding the presence of transcription factors. However, several transcription factors such as C/EBPs, SREBP, and PPARs were previously shown to regulate both the human and mouse adiponectin promoter, and the binding sites were well characterized (29–31) (Fig. 1). Bioinformatics binding site prediction revealed putative binding sites for the large family of homeodomain proteins and zinc finger proteins, yet clearly no binding sites for the so far known regulators of adiponectin. Although we could clearly show that the

SNPs modulate DNA binding activity, the exact binding factors remain to be identified. A recent publication also suggested SNP3 by bioinformatics prediction to modify a zinc finger protein site (33), but in this work, no attempt was made to analyze the influence of the SNP3 on DNA binding activity.

The correlation between elevated circulating adiponectin levels and the presence of the minor allele in SNP2 is in line with published data and with a recent report of increased promoter activity in COS-7 cells (11). However, COS-7 is not an adipocyte cell line and may not express an appropriate set of transcription factors expected in adipocytes. Direct adiponectin measurement of endogenous adiponectin is not possible in the available cell models, since the cell line does not contain the different genomic haplotypes, but our transfection studies represent a good model to address this aspect.

The minor allele of SNP2 resulted in a higher inducibility by rosiglitazone only in the combination with the major allele in SNP1. Even more for some minor allele constructs, this inducibility was increased from a lower basal level, whereas the haplotype with three minor alleles was not inducible at all. This serves as an additional hint for a functional interaction between these two SNPs and furthermore that a functional analysis of SNPs should also take into account the activation state of cells. Peroxisome proliferator-activated receptor (PPAR)- γ agonists such as rosiglitazone are known to induce adiponectin expression in adipocytes (34,35). Furthermore, treatment of type 2 diabetic patients with rosiglitazone improves insulin sensitivity but stimulates fat accumulation (36). The response to glitazones in humans could possibly differ depending on the promoter haplotype, which pinpoint a potential relevance of the *APM1* promoter SNPs for improved individualized treatment.

We found obvious changes in the promoter and DNA binding activity when the minor allele in SNP1 was present. These findings are in line with the known association of SNP1 with hypoadiponectinemia (9–11) and the significantly lower adiponectin levels in our MONICA/KORA S123 sample. Moreover, the epidemiological haplotype data extend these findings, with adiponectin being downregulated by the minor allele of SNP1 and upregulated by the minor allele of SNP2. An important challenge for the future characterization of this functional module is the identification of the nuclear factors whose binding is affected by SNP1 and SNP2.

We used a combined functional and epidemiological approach and thus were able to overcome the drawback of each approach separately: On the one side, in epidemiological studies, it is not clear whether a significant SNP association is derived from the analyzed SNP directly or from a latent SNP in linkage disequilibrium. Significant haplotype associations can pinpoint a certain haplotype of interest, but it would remain to be shown which specific allele combination—and possibly including latent alleles between genotyped loci—would be of functional relevance. This is overcome by our functional haplotype promoter studies where all effects are clearly attributed to the distinct alterations analyzed, since polymorphisms in linkage disequilibrium have not been mutated; hence a major functional impact is exerted by the combination of the here analyzed SNPs. On the other hand, functional studies alone do not allow the drawback to effects in humans. This limitation is overcome by adding epidemiological data, which support the functional findings regard-

ing the regulation by SNP1 and SNP2, the effects of haplotype combination, and a potential negative selection of the haplotype with minor alleles in all three SNPs due to suppressed adiponectin promoter activity.

In conclusion, the present study on the *APM1* gene is the first one analyzing the functional activity of *APM1* regulatory SNPs in a cell model expressing endogenous adiponectin and shows the importance to consider SNP haplotypes. The epidemiological data support the functional findings and thereby underscore the relevance in humans. Our results demonstrate that promoter variants in the *APM1* gene are relevant for the regulation of adiponectin transcription. Furthermore, our study represents a suitable approach by combining functional and epidemiological methods to characterize the role of gene variants.

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