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Sequencing and haplotype analysis of the Activator of CREM in the Testis (ACT) gene in populations of fertile and infertile males

Greg L.Christensen[1,2], Stephen P.Wooding[3], Ivaylo P.Ivanov[3], John F.Atkins[3,4] and Douglas T.Carrell[1,2,5]

[1]Andrology and IVF Laboratories, [2]Department of Physiology, [3]Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, UT 84108, USA and [4]Biosciences Institute, University College, Cork, Ireland

[5]To whom correspondence should be addressed at: Andrology and IVF Laboratories, University of Utah School of Medicine, 675 Arapeen Drive, Suite 205, Salt Lake City, UT 84108, USA. E-mail: dcarrell@med.utah.edu

cAMP-responsive element modulator (CREM) is a key transcription factor in the differentiation of round spermatids into mature spermatozoa. During spermiogenesis, CREM is regulated in part by activator of CREM in the testis (ACT), which activates CREM in a phosphorylation-independent fashion. We hypothesized that the ACT gene, which is expressed exclusively in the testis, could be involved in male factor infertility in patients with idiopathic-impaired spermatogenesis. To test this hypothesis, we sequenced the coding regions and flanking intronic regions of the ACT gene in 96 azoo- or oligospermic patients and 69 fertile controls. A total of 12 single-nucleotide polymorphisms (SNPs) was identified, and four of them leading to amino acid substitutions. An association study was performed based on calculated haplotype frequencies, and statistically significant differences were found between the patient and control populations for some haplotypes. To help establish the evolutionary relationships between the haplotypes, the coding regions of both the chimpanzee and the gorilla ACT gene were sequenced and evaluated. To test whether the different haplotypes conferred a functional change to the ACT protein, a yeast two-hybrid assay was designed to test the interaction between the two most divergent ACT haplotypes and their known binding partners, CREM and KIF17b. We identified one ACT haplotype that had a 45% reduction in its interaction with CREM. Our results suggest that different haplotypes within the ACT gene may contribute to male factor subfertility.

Introduction

While several causes of male infertility have been revealed, the majority remains idiopathic. With an estimated 2000 genes contributing to spermatogenesis (Hargreave, 2000), it is probable that many genetic causes of impaired spermatogenesis remain undiscovered. With few exceptions, the growing list of male infertility candidate genes identified from transgenic animal studies (Escalier, 2001; Christensen and Carrell, 2002) is poorly characterized in infertile, male, human populations. Identifying which of these genes are of clinical significance will assist in developing microarray technologies to screen for mutations in multiple spermatogenesis genes in a single patient (Russo *et al*., 2003; Rossi *et al*., 2004). The purpose of this study was to screen one of these candidate genes, activator of cAMP-responsive element modulator (CREM) in the testis (ACT), in populations of infertile and fertile males

The ACT gene, located on chromosome 6q16.1–16.3, contains five coding exons and encodes a 284 amino acid protein (Morgan and Whawell, 2000; Palermo *et al*., 2001). ACT is a member of the four-and-a-half LIM (FHL) domain family of proteins. LIM domains are a tandem zinc finger structure that acts as a modular protein-binding surface. The LIM proteins participate in a variety of diverse processes related to cytoskeletal function and the control of gene expression (Kadrmas and Beckerle, 2004). A single LIM domain is approximately 55 amino acids in length and contains eight highly conserved cysteine or histidine residues, at defined intervals, which bind the zinc ions (Schmeichel and Beckerle, 1994). The remainder of the domain is less conserved and likely plays a role in the specificity of the protein–protein interaction (Schmeichel and Beckerle, 1997). The FHL proteins, including ACT, are organized with a half LIM domain (single zinc finger), followed by four double zinc finger domains, and with each domain connected to the next by an eight amino acid linker. The FHL proteins affect gene expression by binding as a cofactor to other transcription factors, such as members of the CREB family (cyclic AMP response element binding protein) (Fimia *et al*., 2000).

The CREM protein exists in different isoforms in different tissues, which can either activate or repress transcription by binding to the cAMP response element (CRE) in the promoter of various genes (Molina *et al*., 1993; Daniel *et al*., 2000). The CREM-τ-activating isoform is the one most abundant in the post-pubertal testis (Sassone-Corsi, 2000). In somatic cells, CREM binds a CRE and activates transcription after it has been phosphorylated at the Ser-117 residue. In the haploid cells of the testis, CREM activation can be initiated when it is bound by the cofactor ACT, independent of phosphorylation (Fimia *et al*., 1999). Several genes including the protamines, transition proteins, calspermin and angiotensin-converting enzyme contain CRE elements in their promoters and are transcribed through CREM activation (Kistler *et al*., 1994; Sun *et al*., 1995; Sassone-Corsi, 1998; Kimmins *et al*., 2004). Homologous disruption of the CREM gene in transgenic mice results in complete spermatogenic arrest (Blendy *et al*., 1996; Nantel *et al*., 1996) while heterozygotes have reduced counts, abnormal morphology and significantly litter sizes (Nantel *et al*., 1996).

The ACT protein also interacts with at least another protein. Two groups independently demonstrated that ACT binds to KIF17b, a kinesin motor protein (Macho *et al*., 2002; Chennathukuzhi *et al*., 2003). It appears that KIF17b plays an indirect role in the regulation of CREM activation by shuttling ACT out of the nucleus and transporting

it into the cytoplasm, where it can no longer activate CREM-dependent transcription (Kotaja *et al*., 2005).

A recent report, in which the details from an ACT-deficient mouse were described, supports the importance of ACT as a contributor to normal spermatogenesis. Mice that had the ACT gene disrupted by homologous recombination had only 35% of the sperm concentration of control mice. In addition, 80% of the sperm tails were folded or bent. The sperm heads also demonstrated highly abnormal morphology (Kotaja *et al*., 2004). While the mice were still fertile, the report demonstrates that ACT plays an important role in the correct formation of male gametes.

To determine whether mutations in the ACT gene account for some cases of male infertility, we screened 96 patients and 69 fertile controls by directly sequencing the exons and flanking intronic sequence of the ACT gene. In addition to screening the ACT gene for possible mutations associated with infertility, we have also examined the frequency of haplotypes that emerged and the evolutionary relationships among the haplotypes. Finally, to examine the relationship between variation in this gene and disease phenotype, we tested for associations between haplotype and phenotype.

Materials and methods

Patients and controls

After obtaining University of Utah institutional review board approval for all aspects of the study, patients with severe male factor infertility were identified and invited to participate. In total, 47 men with azoospermia and 49 men with severe oligozoospermia (<5 million sperm/ml) were enrolled in the study. To increase the chance of identifying a mutation in the ACT gene responsible for male infertility or subfertility and to decrease possible confounding factors, patients were excluded if they had any suspected or known causes of male infertility. For example, patients with a known Y-chromosome microdeletion, cystic fibrosis, varicocele, Klinefelter's, exposure to chemotherapeutics or radiation, etc. were not included. In addition to the infertile population, 69 men of known paternity were also included as controls from the Utah Genetic Reference Project (UGRP). After the patients gave informed consent, venous blood was taken using standard phlebotomy techniques and the genomic DNA extracted using the Puregene DNA extraction kit (Minneapolis, MN, USA). To assess evolutionary relationships with non-human primates, DNA was obtained for both the common chimpanzee (*Pan troglodytes*) and the gorilla (*Gorilla gorilla*).

Screening for mutations

Primer sets were designed and optimized to amplify each of ACT's five coding exons and the flanking intronic sequence from the collected genomic DNA, using standard PCR techniques (Table I). The same primers were used to amplify the human and non-human DNA. Thermocycling conditions were as follows: 94°C for 4 min followed by 35 cycles of 94°C for 30 s, annealing temperature (listed in Table I) for 30 s, base extension 1 min per kb at 72°C and a final hold for 5 min at 72°C. Primary PCR products were cleaned-up using a gaunidine HCL/KOAc protocol and sequenced in the forward and reverse directions on an ABI 3700 capillary sequencer.

Sequence trace files generated from the ABI 3700s were analysed using the Phred, Phrap and Consed software programs (www.phrap.org). Phred assigns a quantitative value to the quality of each sequenced base. This base quality provides a probabilistic estimate of the correctness of the base call. The sequences were assembled with ACT's specific consensus sequence using the Phrap program and potential mutations identified using Consed, which has the ability to search for high-quality base discrepancies, based on the Phred values, in the assembled sequence. A visual analysis was also conducted of the trace files to confirm identified polymorphisms and potential mutations.

Haplotype analysis

Haplotypes were inferred from unphased genotypic data using the PHASE 2.0.2 computer program (Stephens *et al*., 2001), which was run for 100 000 iterations following a burn in of 5000 iterations. These analyses were applied to all nucleotide positions typed in both the patient and the control populations. Evolutionary relationships among the haplotypes were inferred using the Arlequin computer program (Schneider *et al*., 2000), which reports a minimum spanning tree.

A case–control association analysis was performed using the PHASE 2.0.2 computer program, run using the 'case-control' option under the same parameters as for haplotype inference. This analysis tested the hypothesis that the haplotypes were randomly distributed between the patient and control populations. Chi-squared tests were used to test individual haplotypes for over- or under-representation in the case population.

A maximum likelihood phylogeny relating human ACT haplotypes to haplotypes in chimpanzee and gorilla was generated using the PHYLIP 3.6 package (Felsenstein, 1993). As full ACT sequence data were not available for all three species, this phylogeny was based upon cDNA sequence data only.

Yeast two-hybrid screening

Clones containing the cDNA sequences of the CREM and ACT genes (ID numbers 5298443 and 4827311) were obtained from OpenBiosystems (Huntsville, AL, USA). A clone containing the Kif17b cDNA was kindly provided by Norman Hecht, PhD. Using the clones as a template, the CREM activation domain (amino acids 1–229), previously shown to interact strongly with ACT (Fimia *et al*., 1999), was obtained by PCR and cloned into the PMA424 vector (Clontech), adjacent to the GAL4-binding domain. The full open-reading frame of Kif17b was also cloned into the PMA424 vector in frame with the GAL4-binding domain. The full-length coding sequence of ACT was obtained by PCR and cloned into the pGAD424 vector (Clontech), next to the GAL4-activation domain. A 'mutant' version of ACT, containing four of the nucleotide changes identified in the coding sequence (numbers 4, 6, 10 and 11 in Table II), was generated using site-specific mutagenesis PCR techniques.

The pGAL1-LacZ vector, containing a LacZ reporter, and either the PMA424-CREM or PMA424-Kif17b clone were transformed into the YM4271 yeast strain using a high lithium protocol and plated onto the appropriate synthetic dropout agar. Several colonies were selected and cultured, and the wild-type or mutant pGAD424-ACT clone was independently transformed into each one. The β-galactosidase assay was performed, as described previously (Ma and Ptashne, 1987). Briefly, overnight yeast cultures containing the combination of vectors being tested were washed and resuspended in Z buffer and the OD^{600} recorded. The cells were lysed with 0.1% SDS and chloroform, and 500 µl of the solution was aliquoted in triplicate. Fifty microliters of ONPG (4 mg/ml) was added to each aliquot to start the reactions. Once the aliquots began turning yellow, they were stopped with 250 µl of Na_2CO_3 and the OD^{420} was recorded. The activity was calculated in Miller units [1000 × (OD420/volume cells assayed (ml) × time of assay (min) × OD600)] and is the mean of assays performed in triplicate from independent transformations

Results

Mutation screening

A total of 96 oligozoospermic or azoospermic patients and 69 fertile controls was screened for mutations in the coding sequence and flanking

Table I. Primers for activator of cAMP-responsive element modulator (CREM) in the testis (ACT) PCR amplification

Exon	Forward (5′–3′)	Reverse (5′–3′)	Annealing temperature	Product size
1–3	ATACAGGGAACTGCCAAGATG	AAAGATGGTCATGCTCATCG	63°C	2858 bp
4	TAGAGTAGCCAAAAGCACCAGTTG	GTACTCTTCTCCTCCCACTCAA	61°C	715 bp
5	TAGCATTCTGCCTTCTTCTGTTG	CCAGGAATGCAAACACTAGC	61°C	821 bp

Table II. Nucleotide variations detected in the human activator of cAMP-responsive element modulator (CREM) in the testis (ACT) gene

Number	Region	Nucleotide change	Amino acid change	NCBI	Patients						Controls (n = 69)	P*	All patients versus controls
					Azoospermic (n = 47)		P*	Oligospermic (n = 49)		P*			
1	Exon 1	103 G/A	R35H	New	G .97	A .03	–	G .98	A .02	–	N/A		–
2	Exon 2	1264 C/T	N96N	rs2064947	C .85	T .15	–	C .91	T .09	–	N/A		–
3	Exon 4	6987 T/C	C178C		T .99	C .01	NS	T .99	C .01	NS	T .99	C .01	NS
4	Exon 4	7063 A/G	R204G	New	A .75	G .25	.0443	A .77	G .23	.0168	A .62	G .38	.0072
5	Exon 4	7077 A/G	P208P	New	A .85	G .15	NS	A .91	G .09	NS	A .83	G .17	NS
6	Exon 4	7084 A/G	M211V		A .4	G .6	NS	A .52	G .48	.018	A .33	G .67	.0336
7	Intron 4	7222 A/G	–	New	A .99	G .01	NS	A .98	G .02	NS	A .98	G .02	NS
8	Intron 4	7259 A/G	–	New	A .99	G .01	NS	A .98	G .02	NS	A .98	G .02	NS
9	Intron 4	7441 T/C	–	New	T .97	C .03	NS	T .97	C .03	NS	T .98	C .02	NS
10	Exon 5	12032 C/G	S243R	rs9373985	C .75	G .25	NS	C .78	G .22	.023	C .64	G .36	.0154
11	Exon 5	12065 A/G	L254L	rs9398148	A .75	G .25	NS	A .78	G .22	.023	A .64	G .36	.0154
12	3′ UTR	12234 T/G	–	rs9398152	T .94	G .06	.0058	T .9	G .1	.0322	T .81	G .19	.0023

NS, non-significant.
*Statistical analysis for patients versus controls.

intronic sequence of the ACT gene by direct sequencing. A total of 12 single-nucleotide polymorphisms (SNPs) was identified within the gene (Table II). Four of the SNPs have been reported in the NCBI database previously, and two more reported in a publication (Palermo *et al*., 2001). This is the first study in which the frequencies of the new and previously identified polymorphisms have been reported. It is of note that several other variants recorded in the NCBI SNPs database or reported by Palermo *et al*. (2001) were not found.

Eight of the 12 SNPs observed fell within the coding regions of the ACT gene. Four of these, including two not previously reported, resulted in amino acid changes. A chi-square test was performed to determine if there was a significant difference in the occurrence of the different alleles in the patient populations and the control population. One SNP, 7063 A/G, was significantly different than the controls in the azoospermic population. When the oligozoospermic population was compared with the controls, five SNPs, numbers 4, 6, 10, 11 and 12 (Table II), showed significant differences between the populations. When all 96 infertile patients were compared together with the control group, the P value for four of these five SNPs was increased.

The position of each amino acid changing SNP was evaluated in relation to its location in the protein. The first change, *R35H*, is a conservative change from arginine to histidine and falls within the first eight amino acid linker connecting the half LIM domain at the beginning of the protein with the first whole LIM domain. These linker regions do not appear to be highly conserved. Amino acid changes *R204G* and *M211V* fall within the third full LIM domain, and *S243R* is in the fourth and final LIM domain. All three of these SNPs result in a change of either the charge or the polarity of the affected amino acid. None of these three changes disrupt the cysteine residues critical for binding zinc, however, they do fall within the regions considered important for protein interactions. Although these three amino acid changes were common in both the patient and the control populations, there were significant differences in the frequencies of all three changes between the groups.

Haplotype analysis

Analyses of the case and control genotypes using PHASE revealed 14 haplotypes (Table III). These ranged in frequency from <0.01 to ~0.30 in the case sample and from <0.01 to ~0.43 in the control sample.

The minimum spanning tree relating the haplotypes revealed that variation in our sample was dominated by the presence of two common, closely related haplotypes, 10 and 12, which together accounted for nearly 40% of all observations (Figure 1). However,

Table III. Haplotype frequencies estimated for patient and control groups

Number	Haplotype*	Patients		Controls	
		Number	Frequency	Number	Frequency
1	TGGGAATGGT	23	0.120	17	0.123
2	TGGGAATGGG	0	0.000	9	0.065
3	TGAGAATGGT	1	0.005	6	0.043
4	TGAGAATGGG	16	0.083	15	0.109
5	TGAGAATCAT	0	0.000	1	0.007
6	TGAGGGTGGG	0	0.000	1	0.007
7	TGAGGGCGGT	3	0.016	0	0.000
8	TGAGGGCGGG	0	0.000	2	0.014
9	TAAGAATGGT	0	0.000	1	0.007
10	TAAGAATCAT	62	0.323	40	0.290
11	TAAGAACGGT	1	0.005	0	0.000
12	TAAAAATCAT	84	0.438	44	0.319
13	TAAAAATCAG	0	0.000	1	0.007
14	CGAGAACGGT	2	0.010	1	0.007
		192	1.000	138	1.000

Variant order coincides with single-nucleotide polymorphisms (SNPs) 3–12 in Table I.

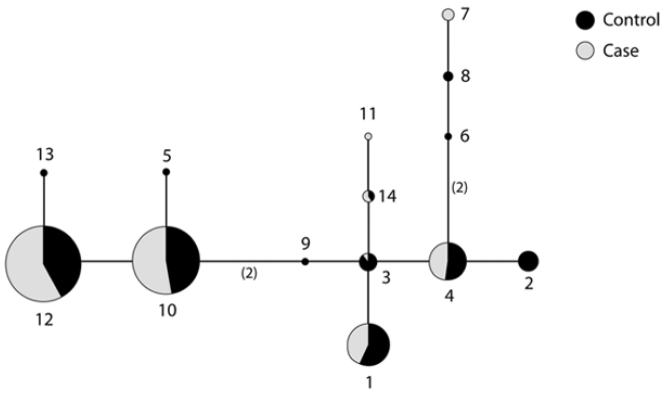

Figure 1. A diagram of a minimum spanning haplotype network that shows the putative evolutionary relationships among the inferred haplotypes. Each circle represents a haplotype, and the area of the circle represents the haplotype's relative frequency. Lines between circles represent single-nucleotide differences, except where indicated in parentheses. The shading indicates the relative frequency of the haplotype in each population. For example, a circle that is shaded exactly half black and half grey indicates that the haplotype was equally likely to be found in the case and control populations, when sample size differences are taken into account.

several rare, evolutionarily divergent haplotypes were also present. Two (haplotypes 1 and 4) were moderately common and found at frequencies of 0.12 and 0.09, respectively.

On the basis of the observed genotypes, 14 haplotypes were estimated to be present in the infertile and fertile study groups, using SNPs 3–12. The first and second SNPs were not included in the haplotype analysis because of incomplete data for the control group. Haplotypes 10 and 12 were the most frequently observed, accounting for 76% of the infertile group and 61% of the fertile group. An analogue of the Fisher's exact test was run using Phase 2.0.2 to determine whether the haplotypes were uniformly distributed between the fertile and infertile populations. This case-control analysis rejected the hypothesis that, in aggregate, haplotypes were randomly distributed between the case and control populations ($P < 0.03$). This is most likely because of the decrease of haplotypes two and three and the increase of haplotype 12 in the patient group.

Non-human primate analysis

To gain insight into how or when the different haplotypes may have evolved, we assembled the ACT cDNA sequence of the chimpanzee and gorilla. The chimpanzee and gorilla sequence differed by eight and four nucleotides, respectively, from the most common human haplotype (12). The gorilla also had an additional four heterozygous nucleotide variants, with one allele the same as humans and the others unique to the gorilla. The chimpanzee had five amino acids and the gorilla two amino acids that were different from haplotype 12. Only one of these, 211V, was also variable within humans.

Yeast two-hybrid

It was initially observed that SNPs 4, 6, 10 and 11, which included the three most common amino acid changes, segregated together into three haplotypes, referred to as A, B and C (Table IV). With the exception of one patient and two controls who demonstrated recombination in one of their two alleles, all samples tested could be divided into one of these three haplotypes, which contained none, one or three amino acid differences. Chi-square analysis indicated that the distribution of the three haplotypes between the patients and controls was significantly different ($P = 0.02$). To determine whether the three amino acid changes present in haplotype C conferred a functional change to ACT, a yeast two-hybrid assay was designed to test the interaction between either the CREM activation domain or the Kif17b and haplotype A or C of the ACT protein (Table V). Four independent assays, performed in triplicate, were tested for each combination. These assays showed that the binding activity for CREM–ACT C was significantly lower than for CREM–ACT A, a 45% reduction on average ($P < 0.01$, χ^2). Small fluctuations in binding activity were observed between Kif17b and haplotypes A or C. However, they did not follow a consistent pattern, with A or C being alternatively higher, and were not of statistical significance. As multiple factors including time, ONPG concentration and plasmid copy number can affect Miller unit values, tests of haplotypes A and C with CREM or with Kif17b were run concurrently and only values for the same assay compared.

Table IV. Haplotype frequencies for single-nucleotide polymorphisms (SNPs) 4, 6, 10 and 11

Haplotype	Amino acid/nucleotide	Patients ($n = 96$)	Controls ($n = 69$)
A	204R-211M-243S-*12065A*	0.45	0.33
B	204R-211V-243S-*12065A*	0.31	0.29
C	204G-211V-243R-*12065G*	0.24	0.38

Table V. Yeast two-hybrid activity levels

	Assay	Miller units	
		ACT A	ACT C
LacZ-CREM	1	139	55
	2	124	98
	3	121	60
	4	83	46
LacZ-Kif17b	1	277	254
	2	201	228
	3	149	183
	4	270	164

CREM, cAMP-responsive element modulator.

Discussion

In this study, we conducted a mutation screen of the ACT gene in 96 azoospermic or severely oligospermic patients and 69 fertile controls. We identified 12 nucleotide changes, including four that yield an amino acid change, by direct sequencing. All of the identified nucleotide changes were present in both the infertile patient population and the fertile controls, suggesting that they do not individually contribute to the pathogenesis of male infertility. However, the frequency of the nucleotide changes, and the distribution of some of the associated haplotypes, did show significant differences between the patients and controls.

The patterns of haplotype diversity we identified in the ACT gene indicate that most genetic variation is accounted for by just four haplotypes (1, 4, 10 and 12). In addition, the minimum spanning tree reveals that these haplotypes fall into two groups, one (Group A) consisting of haplotypes 5, 10, 12 and 13 and the other (Group B) consisting of all other haplotypes. It should be emphasized that because our sample is composed exclusively of individuals of European ancestry, that our sample is only representative of European populations. Other populations may harbour different SNPs and haplotypes.

Evidence from the haplotype–based association analysis suggests that variation in this gene may be correlated with a disease phenotype. Overall, haplotypes are not distributed uniformly between the patient and control populations, and the patient population harbours several rare haplotypes not observed in controls. Further, two haplotypes (2 and 3) were found at significantly elevated frequencies in the controls, and one, haplotype 12, was significantly underrepresented. Interestingly, the first two haplotypes are found in Group B while the latter is found in Group A. The presence of two positively associated haplotypes in Group B, along with the presence of a negatively associated haplotype in Group A, suggests the hypothesis that there may be some difference in susceptibility associated with groups A and B. A rigorous test of this hypothesis will require more intensive sampling.

A key difference between groups A and B is the distribution of the amino acid changing variations. Group B always has the same three variations: 204G, 211V and 243R, whereas Group A has 204R, 243S and either 211M or 211V. Yeast two-hybrid assays demonstrated that there is a decrease in binding affinity between ACT and CREM for Group B, compared with Group A. However, because both major haplotype groups are found in the patient and control populations, the presence of the Group B haplotypes alone are also unable to account for the patient group's infertility. This is further supported by data from the ACT knockout mouse, which showed that even mice with a complete absence of ACT are still fertile, though spermatogenesis is impaired (Kotaja *et al*., 2004).

An analysis of the non-human primate sequence showed that, with respect to the cDNA sequence, the gorilla and chimpanzee are more

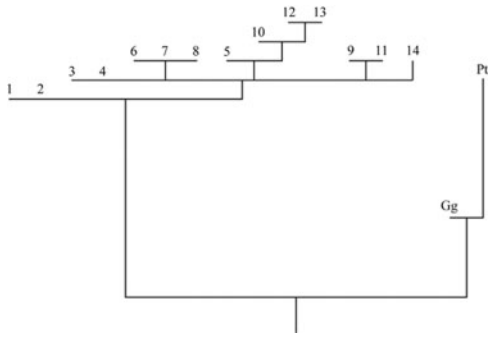

Figure 2. Maximum likelihood phylogeny-relating human, chimpanzee (Pt) and gorilla (Gg) cDNA sequences. All human sequences are more closely related to each other than any is to chimpanzee or gorilla. Chimpanzee and gorilla are more similar to each other than either is to human, although gorilla is somewhat more similar to human than is chimpanzee.

similar to the Group A haplotypes, having the 204R, 211V and 243S variations. This implies that the Group B haplotypes may have evolved from Group A. The maximum likelihood phylogeny-relating human, chimpanzee and gorilla cDNA sequences revealed that the human and gorilla sequences were more similar to each other than were human and chimpanzee sequences (Figure 2). This result stands in contrast to results suggesting that humans are, overall, more similar to chimpanzees than to gorillas, although not all individual genes show this pattern (Salem *et al*., 2003). Such patterns arise often by chance through stochastic processes; however, an alternative explanation could be that natural selection has caused high rates of nucleotide substitution in humans and chimpanzees relative to gorillas (Bamshad and Wooding, 2003).

Because of the complexity of male infertility, it has been argued that candidate genes will need to be screened directly, rather than relying on mapping studies, to determine which are involved in impaired spermatogenesis (Gianotten *et al*., 2004). Several studies have attempted to identify causative mutations for infertility, with limited success (Rovio *et al*., 2001; Liao and Roy, 2002; Gianotten *et al*., 2003; Olesen *et al*., 2003; Christensen *et al*., 2005). In this study, we have identified 12 SNPs in the ACT gene. Several of these appear at significantly different frequencies in the fertile and infertile groups evaluated. Though none of the identified SNPs appear independently causative for male infertility, evidence supports the idea that SNPs involved in complex traits, like infertility, generate amino acid changes in less-critical areas of the protein. A number of the complex trait-associated SNPs, spread over several genes, can then combine to generate a phenotype (Thomas and Kejariwal, 2004). Viewed from this perspective, some of the identified ACT SNPs may play a role in male infertility, when combined with SNPs in other genes.

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