RESEARCH ARTICLE

GATA/GACA repeat sequences are transcribed in the normal fertile rat *Rattus norvegicus*, but not in the infertile ones

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Repetitive DNAs in higher eukaryotes have been implicated with genome organization, chromatin conformation and gene regulation. We studied tissue- and stage-specific expression of 18 nt long $(GATA)_{4.5}$ and $(GACA)_{4.5}$ sequences in somatic tissues and germline of normal fertile rat, including samples from individual testicular cell types and those of adult infertile animals. Barring heart, all somatic tissues showed moderate-to-high level of expression with GACA. Amongst testicular cell types, only Sertoli cells showed a strong signal with GACA, and a faint one with GATA probe. RNA samples from different somatic tissues and testis of infertile rats did not show hybridization with these probes. Database search showed 100% sequence homology of $(GATA)_{4.5}$ with polymorphic loci in 19 different species, whereas $(GACA)_{4.5}$ showed 100% homology with transcribing sequences in 37 different species. In addition to most of the somatic tissues, expression of GATA/GACA elements exclusively in Sertoli cells but not in ovary, suggests their possible involvement in regulation of gonadal activities in the males. This observation is corroborated by the lack of transcription of GATA/GACA repeats in the somatic tissues and testes of infertile Brown Norway rat. The ubiquitous expression of these sequences in somatic and germline tissues of the normal rat but not in any of the tissues of proven infertile rat, indicates their possible regulatory role in the male gonad.

IN most of the evolved vertebrates, a major part of the sex chromosome contains satellite DNA sequences[1]. One such satellite DNA fraction, *Bkm* (banded krait minor), was isolated from the W-chromosome of snakes *Bungarus fasciatus*[2] and *Elaphe radiata*[3]. Subsequent analysis revealed that the *Bkm* fraction predominantly contains GATA/GACA simple repeat motifs[3]. Since then, extensive studies have been conducted to uncover its genomic distributions and possible biological functions in a number of species, particularly in the light of their association with the heteromorphic sex chromosome[4]. *In situ* hybridization of the human and non-human metaphase chromosomes using *Bkm*[5], its sub-fragments[6] and pure synthetic GACA/GATA oligonucleotide probes[7] revealed their ubiquitous presence on the chromosomes, with preferential localization in the heterochromatic regions of X-chromosome in some vertebrates[8]. In mouse testis and somatic tissues, *Bkm*-related GATA/GACA sequences have been reported to be transcribing[3,9]. Similarly, cDNA library screening has shown these sequences to be part of several transcribing genes[10]. Based on genomic organization and expression studies[3,7,9–11], GATA/GACA sequences have been implicated in directing the events leading to vertebrate sex determination[3,5,9]. However, no report is available on their tissue- and stage-specific expression in any vertebrate. Similarly, information on expression in response to environmentally varying temperatures or 'heat shock' is not available, though the latter has been reported to modulate alternate splicing of the functional genes[12]. These aspects are of biological interest because temperature seems to alter the sex of some vertebrates (e.g. turtles), albeit without throwing light on the oblivion of the mechanism of sex determination. In view of these reports and lack of information on the expression of GATA/GACA repeats in infertile animals, we studied their overall somatic and gonadal expression during the course of development in the normal fertile and adult infertile rats. Expression was also monitored independently in spermatogonia, round spermatids, pachytene spermatocytes and Sertoli cells, employing Northern blot hybridization. In response to environmental temperature, expression was studied in the total RNA isolated from normal adult testis and the one surgically embedded in the abdomen for 72 h. Finally, GenBank database was searched for the presence of 18 nt GACA and GATA repeats within the transcribing genes from different species. Possible involvement of these transcribing satellite sequences in the regulation of spermatogenesis in rats is discussed.

Materials and methods

Total RNA isolation

Total RNA was isolated from the rat testes of day 3, 5, 6, 7, 8, 9, 10, 11, 13, 15, 17, 20, 30, 40 and adult (day 70) using Tri-X reagent kit (Molecular Research Center,

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Cincinnati, Ohio) according to the supplier's specifications. RNA from rat type-A spermatogonia, Sertoli cells, pachytene spermatocytes and round spermatids was obtained from independent cultures. Rat type-A spermatogonia and Sertoli cells were isolated following the established methods[13,14] and pachytene spermatocytes and round spermatids by centrifugal elutriation[15]. In addition, RNA from adult rat was also isolated from kidney, spleen, muscle, lung, liver, heart, brain and ovary following the standard protocol[16]. Five proven infertile Brown Norway male rats were included in the study for total RNA isolation from somatic tissues and testes following the above-mentioned procedure. All animal experiments were conducted according to protocols approved by local Institutional Animal Care and Use Committee.

Histological examination

To substantiate infertility status of the rats used subsequently for expression studies together with the normal ones, testes from five infertile Brown Norway rats were removed, fixed in Bouins solution, embedded in paraffin and serial cross-sections of 5 µM were obtained. Slides were stained with hematoxylin and eosin and photographs were taken following standard procedure[17].

Electrophoretic separation of RNA and Northern blot hybridization

Approximately 10 µg of total RNA representing the above-mentioned samples from somatic tissues and germline and from specific cell types of testes were electrophoresed on 1% agarose gel with formaldehyde. The gel was stained with ethidium bromide and photographed under UV. RNA was transferred onto the nylon membrane, immobilized by UV Strata-linker (Stratagene, San Diego, USA) and hybridized with labelled oligodeoxyribonucleotide probes $(GATA)_{4.5}$ and $(GACA)_{4.5}$, following standard procedure[16].

Densitometric analysis and quantitation of mRNA transcripts

All the RNA blots hybridized with oligo probes were analysed on an optically enhanced densitometer, 420oe, with 42 µm resolution in the range of 400 750 nm wavelength using 'Diversity One' software (PDI, Inc., New York, USA). For size estimation, 28S and 18S rRNA bands were taken to be 5.2 kb and 2.2 kb, respectively and the relative size of the discernible bands was determined directly both in kb and 'S' unit using the above-mentioned software. Based on optical density, signals detected in different RNA samples from various tissues, including testis during different stages of development were estimated. Finally, bands in all the lanes were plotted to obtain a comparative account of the signals in different tissues. The level of GATA/GACA expression in the testis embedded in the abdomen and the same from a normal one was quantitated using the above-mentioned densitometer.

Results

The rationale of using 18 nt long oligonucleotide as hybridization probe for expression studies is based on the established parameter[18].

Expression of $(GATA)_{4.5}$/$(GACA)_{4.5}$ sequences in rat somatic tissues and ovary

Northern blot analysis with the $(GATA)_{4.5}$ probe revealed weak signals in most of the somatic tissues but a strong signal in the kidney, whereas no signal was detected in the heart and muscle (Figure 1). The $(GATA)_{4.5}$ probe failed to detect signals in the ovary (data not shown). A minor difference in the level of expression was detected with the $(GATA)_{4.5}$ probe between the total RNA isolated from normal rat testes and the one embedded in the abdomen. The embedded sample showed relatively stronger signal compared to that of the normal one. With the $(GACA)_{4.5}$ probe, varying degrees of signal in different tissues were detected, except in the ovary and heart. The muscle and liver showed weak signals, whereas the kidney, lung, spleen

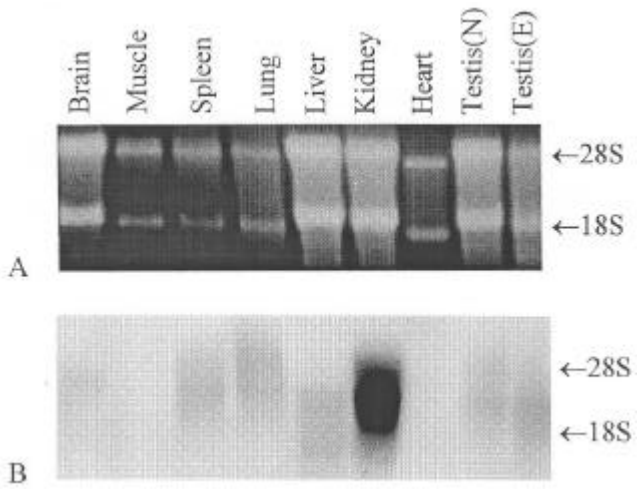

Figure 1. Expression of $(GATA)_{4.5}$ repeats in different somatic tissues and testes based on Northern blot hybridization using total RNA. Panel A denotes ethidium bromide-stained gel and panel B, the autoradiogram. Testes (N) and (E) represent RNA samples from the normal and the one embedded in the rat abdomen for 72 h, respectively. Note the weak signal in testes, negligible ones in brain, absence of the same in muscle and heart and strong signal in kidney.

and testis showed strong signals giving rise to 2–3 discernible bands in the range of 1.9–7.0 kb (Figure 2). Quantitation of the signals confirmed maximum expression in the kidney with an OD value of 2.83, whereas the minimum was seen in the muscle with an OD of 0.74 after nullifying the background.

Expression of $(GATA)_{4.5}$/$(GACA)_{4.5}$ sequences in testis during different stages of development and in individual testis cell types

Testes RNA isolated from different stages of development showed varying levels of expression with the $(GACA)_{4.5}$ probe. No signal was detected in the samples isolated from the testes of day 3, 5, 30 and 40, whereas prominent signals were detected in the samples of day 8, 11, 17 and 70 (Figure 3). Different bands of the same day showed varying signal intensity. Of the four cell types from testes, strong signal was seen only in Sertoli cells with the $(GACA)_{4.5}$ probe (Figure 3, lane SC). The $(GATA)_{4.5}$ probe also detected a faint signal in Sertoli cells, but not in any of the other testicular cell types or in the testes RNA samples isolated during different stages of development (not shown).

Expression of $(GATA)_{4.5}$/$(GACA)_{4.5}$ sequences in the infertile Brown Norway male rats

The histological examination of the infertile rat testis was conducted together with that from the normal fertile one as a positive control (Figure 4 *a* and *b*). The

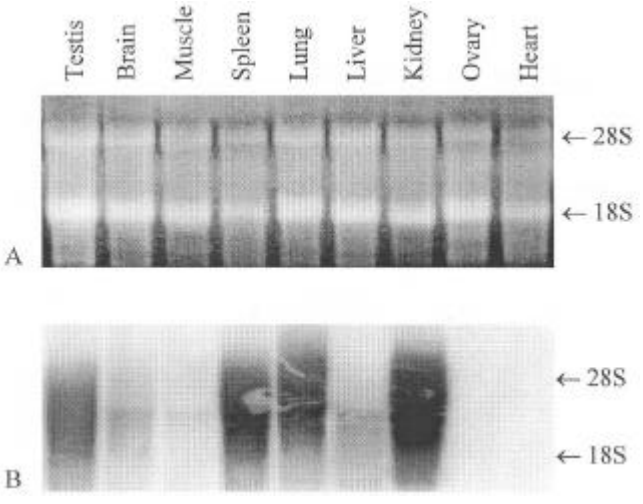

Figure 2. Expression of $(GACA)_{4.5}$ repeats in different somatic tissues, ovary and testis based on Northern blot hybridization using total RNA. Panel A denotes ethidium bromide-stained gel and panel B, the autoradiogram. Note the weak signals in muscle and liver and absence of the same in heart and ovary. Faint signals were also detected in the RNA isolated from testis (N) and (E) hybridized separately (not shown).

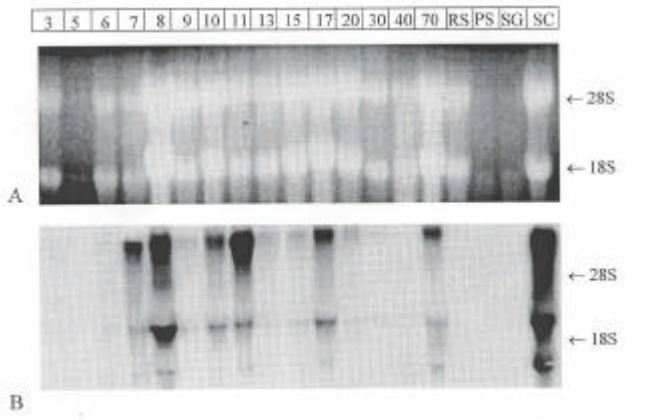

Figure 3. Expression of $(GACA)_{4.5}$ repeats based on the Northern blot hybridization of total RNA isolated during different stages of development (day 3–70) in testes and individual testicular cell types, viz. round spermatids (RS), pachytene spermatocytes (PS), spermatogonia (SG) and Sertoli cells (SC). Panel A denotes ethidium bromide-stained gel and panel B, the autoradiogram.

infertile rat testis showed degenerated seminiferous tubules with a large lumen completely devoid of spermatozoa (Figure 4 *c* and *d*), corroborating its infertility status. GATA and GACA sequences were found to be transcriptionally inactive in all the tissues of the infertile rats, including the testis (not shown).

Discussion

Repetitive sequences have been suggested to be involved in the regulation of gene expression[19]. Transcriptionally active GATA/GACA repeats, associated with the heterochromatic sex chromosome have been implicated with condensation and decondensation cycle of the mouse Y-chromosome[20], suggesting their possible regulatory roles. In the present study, only $(GACA)_{4.5}$ repeats were found to be transcribing in rat testis during most of the developmental stages and in Sertoli cells, whereas $(GATA)_{4.5}$ failed to detect signals in any of the testes samples, except faint signals in the pure Sertoli cells. The low level of $(GATA)_{4.5}$ expression in Sertoli cells corroborates the faint signal detected in the total RNA isolated from the rat testes. Interestingly, a *Bkm*-related clone, m34, containing 32 copies of GATA and present in several species of mouse has been reported to be absent in rat and human[20]. However, faint signals detected in our study suggest that these genomes are not devoid of $(GATA)_{4.5}$ sequences (not shown) which is also corroborated by database search (http://www.ncbi.nlm.nih.gov/blast) showing rat genome positive with GATA repeats. The strong expression of $(GACA)_{4.5}$ and a mild one of $(GATA)_{4.5}$ repeats exclusively in Sertoli cells and absence of the same in other cell types such as round spermatids, pachytene spermatocytes and spermatogo-

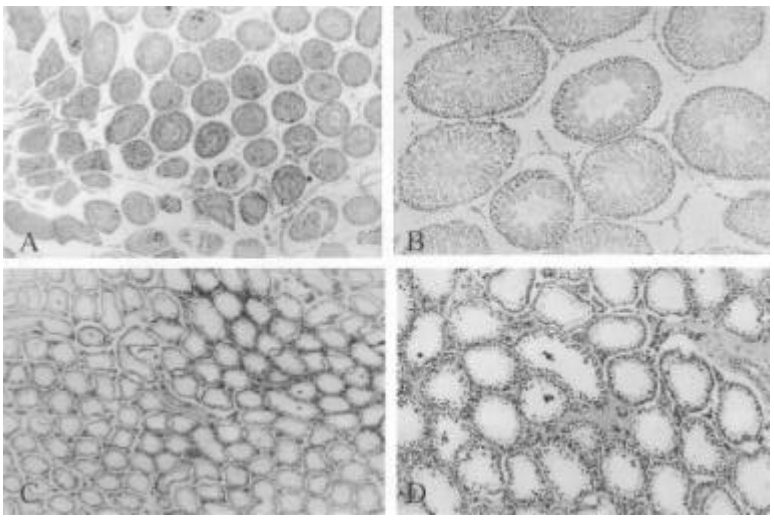

Figure 4. Representative testicular histological sections of the Brown Norway rats. Cross-section of fertile rat testis showing normal morphology, at 10× (***a***) and 40× (***b***). Section of the infertile rat testis showing completely degenerated seminiferous tubule, 10× (***c***) and 40× (***d***).

nia, suggest their possible tissue and/or cell-specific function(s) in the rat genome. Since their discovery, GATA/GACA sequences have been thought to be involved with the events leading to sex determination and differentiation of gonad[20,21]. Lack of expression of these sequences in the infertile rat testes suggests their possible involvement in the gonadal gene regulation. If these sequences are construed to play gonad (male?)-specific roles, their expression in somatic tissue is unwarranted. It is even more startling that the kidney, a non-reproductive organ, shows strong expression of GACA repeats. It is likely that some tissue-specific gene(s), not necessarily involved with gonadal gene regulation, are expressing at a higher level in this tissue. The argument of tissue- or cell-specific functions on one hand, and a generalized role of these sequences on the other hand, both, within the same genome is contradictory. In this context, it may be noted that germ cells are the *raison d'être* for gonads and whether the same become sperm or oocytes depends on the signals from somatic cells, which so far have remained a mystery[22]. It may be noted that a number of genes are expressed predominantly in the testis, but they also show weak expression in other somatic tissues[23,24]. Thus, it is not unreasonable to infer that a number of genes are involved, orchestrating the symphony of spermatogenesis.

Correlation of GATA/GACA sequences with heterochromatin and its transcription

In earlier studies, GATA/GACA sequences have been implicated with heterochromatization (transcriptional inactivation) of the sex chromosome[20]. Despite their putative involvement in heterochromatization, the present study suggests that they may still transcribe under the influence of other regulatory or flanking sequences. GATA/GACA sequences indeed transcribe as part of several genes, which is corroborated by smeary signals in Northern blot hybridizations. Whether these sequences are clustered in the rat genome as a chunk of heterochromatin or organized as part of ORFs, remains inconclusive. Involvement of these sequences with heterochromatization and their expression seems to be analogous to a transcriptionally active light gene of *Drosophila melanogaster*, that is present within the heterochromatin[25]. In related species, the same gene located in euchromatic regions does not transcribe, supporting the regulatory role of repetitive DNA[26]. Thus, the regulatory role of GATA/GACA sequences in the rat genome may not be ruled out.

Biological significance of GATA/GACA sequences

The *Bkm* elements are reported to be present along the entire length of the W-chromosome in snake[27], in the sex-determining (short arm) region and along the entire long arm of the Y-chromosome in mouse[9,28]. Though not many genes have been described from the long arm of the Y-chromosome, its partial deletions are associated with increasing defects in sperm morphology[29,30]. This suggests biological significance of GATA/GACA sequence in the regulation of expression of several still uncharacterized gene(s) involved in normal spermatogenesis. Studies on tissue- and stage-specific expression

of GATA/GACA repeats in other vertebrates would provide a more generalized view of the functional significance of these sequences in the higher vertebrates.

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