ELECTRONIC LETTER

Appraisal of genetic and epigenetic congruity of a monozygotic twin pair discordant for schizophrenia

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The origin of monozygotic (MZ) twins is attributed to two or more daughter cells of a single zygote undergoing independent mitotic divisions, leading to independent development and births. It provides the foundation for the assumption that the resulting twins will be genetically identical. This assumption has been used to evaluate the relative role of genes and environment for a variety of multifactorial features and diseases, including schizophrenia.[1] The interpretation of such results, however, has invariably involved controversy, questioning a number of basic assumptions about intrauterine environment and genetic identity of twins, among others.

In this context, although differences in the placenta, amniotic sac, and vascularisation of separate cell masses can lead to discordance,[2] it is difficult to establish it experimentally.[3] On the other hand, it has been possible to assess to a limited degree the genetic identity of MZ twins, using cytogenetic and genetic technologies. The results have allowed Hall and Lopez-Rangel[4] and others to suggest the involvement of mosaicism in causing discordance between MZ twins. Interestingly, karyotype discordance between monozygotic twins has been reported in a number of genetic syndromes. These include Ulrich-Turner syndrome,[5] Duchenne muscular dystrophy,[6] and Turner syndrome.[7] Molecular differences between rare monozygotic twins have also been reported with respect to telomere length,[8] loss of heterozygosity mutations,[9] triplet repeat expansion,[10] gene expression,[4] HERV[11] and HTLV-1[12] repeat sequences among others. Although such reports suggest possible genomic discordance between the MZ twins, the question remains as to whether they represent an exception or the rule across MZ twin pairs. Also, is genomic discordance a common feature of every pair of monozygotic twins or is it restricted to a few rare cases only? Determination of whether a pair of MZ twins is truly genetically identical would involve a comprehensive analysis. Although this option is not practical given current technologies, genome scanning methods offer a viable option for assessing potential differences between two genomes.

To date, a single study has attempted to identify differences between MZ twins using such an approach.[13] Tsujita *et al*[13] used restriction landmark genome scanning (RLGS), and reported two restriction fragment differences between a single pair of MZ twins. The identity and relevance of the RLGS fragments, however, has not been established. In this research we have used representational difference analysis (RDA), involving six diverse genomic representations, to assess genetic differences in a pair of MZ twins discordant for schizophrenia. The results show that genomic discordance is not present between this pair of MZ twins. This supports the logic that phenotypic discordance of MZ twins is more likely to be the result of non-genetic factors, unless proven otherwise.

MATERIALS AND METHODS

Sources of DNA

Leucocyte genomic DNA was extracted from a number of people with no history of mental disorder and a pair of MZ brothers discordant for schizophrenia (N3 affected, N4 normal). This twin pair was recruited as part of the National Institute for Mental Health (NIMH) twin study as described in Torrey *et al*[14] and the diagnosis was based on DSM-III-R criteria. Born to a 26 year old mother after an uneventful pregnancy, both were delivered by breech four minutes apart. Family history did not include mental illness and both twins finished high school. The first symptoms of the disease surfaced in N3 a few months before his 21st birthday, with

Key points

- Rare monozygotic twins are regularly used in the assessment of genetic *v* non-genetic determinants of complex diseases and phenotypes with the assumption that they have identical DNA sequences. This critical assumption, however, has been questioned in more recent published reports based on reports of cytogenetic differences between some MZ twin pairs. To date, no significant attempt has been made to assess the DNA identity of any MZ twin pair.
- In theory, a number of molecular methods, particularly representational difference analysis (RDA), offer an opportunity to assess the true genetic and epigenetic identity of any pair of MZ twins. Such an assessment is needed, to interpret judiciously the extensive studies on discordance of MZ twins and forms the focus of this report. Our study deals with appraisal of genomic DNA from leucocytes of a pair of monozygotic twins discordant for schizophrenia and involved a series of experiments to develop and test the sensitivity of the technology involving non-human introduced DNA fragment, as a single copy per genome.
- The RDA protocol developed was applied to the twin DNA. Each member was used as tester as well as driver in a pair of RDA experiments carried out in three cycles. It required 14 different RDA experiments using a number of restriction enzymes that generated seven different representations. With the exception of *Hpa*II, which may generate differences in representations because of methylation, all other restriction enzymes are expected to identify sequence variations.
- By estimating the proportion of the human genome assessed in each of the seven representations and using simple probability calculations, we estimate that our experiments have appraised a significant proportion (>50%) of the entire genome, but identified no difference. Such a comprehensive genomic evaluation has not been undertaken before and supports the assertion that the common discordance of MZ twins for a variety of diseases including schizophrenia could not be attributed to their true genomic discordance.

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Table 1 Sequences of adaptors for five restriction enzymes

Primer set	Primer name	Sequence
*Hpa*II A	R Hpa24‡	5′-AGCACTCTCCAGCCTCTCACCGAC-3′
	R Hpa11‡	5′-CGGTCGGTGAG-3′
*Hpa*II B	N Hpa24‡	5′-AGGCAACTGTGCTATCCGAGGGAC-3′
	N Hpa11‡	5′-CGGTCCCTCGG-3′
*Hpa*II C	J Hpa24‡	5′-ACCGACGTCGACTATCCATGAAAC-3′
	J Hpa11‡	5′-CGGTTTCATGG-3′
*Mbo*I A	1M22§	5′-ACCGACGTCGACTATCCATGAA-3′
	1M11§	5′-GATCTTCATGG-3′
*Mbo*I B	2M22§	5′-CTGATCGATACCAGTGCAGATA-3′
	2M11§	5′-GATCTATCTGC-3′
*Mbo*I C	3M22§	5′-CAGTACAGACTGCATCTGAGAT-3′
	3M11§	5′-GATCATCTCAG-3′
*Hin*dIII A	R Hind24*	5′-AGCACTCTCCAGCCTCTCACCGCA-3′
	R Hind12*	5′-AGCTTGCGGTGA-3′
*Hin*dIII B	N Hind24*	5′-AGGCAGCTGTGGTATCGAGGGAGA-3′
	N Hind12*	5′-AGCTTCTCCCTC-3′
*Hin*dIII C	J Hind24*	5′-ACCGACGTCGACTATCCATGAACA-3′
	J Hind12*	5′-AGCTTGTTCATG-3′
*Bam*HI A	R Bam24†	5′-AGCACTCTCCAGCCTCTCACCGAG-3′
	R Bam12†	5′-GATCCTCGGTGA-3′
*Bam*HI B	N Bam24†	5′-AGGCAACTGTGCTATCCGAGGGAG-3′
	N Bam12†	5′-GATCCTCCCTCG-3′
*Bam*HI C	J Bam24†	5′-ACCGACGTCGACTATCCATGAACG-3′
	J Bam12†	5′-GATCCGTTCATG-3′
*Eco*RI A	R Eco24†	5′-AGCACTCTCCAGCCTCTCACCGAG-3′
	R Eco12†	5′-AATTCTCGGTGA-3′
*Eco*RI B	N Eco24†	5′-AGGCAACTGTGCTATCCGAGGGAG-3′
	N Eco12†	5′-AATTCTCCCTCG-3′
*Eco*RI C	J Eco24†	5′-ACCGACGTCGACTATCCATGAACG-3′
	J Eco12†	5′-AATTCGTTCATG-3′

*Lisitsyn *et al*.[15] †Baldocchi and Flaherty.[16] ‡Ushijima *et al*.[17] §This report.

first hospitalisation and diagnosis of catatonic schizophrenia one year later. In the ensuing 15 years, N3 was admitted to hospital on five occasions. On these visits, his diagnosis has consistently been chronic undifferentiated schizophrenia, apart from one diagnosis of schizoaffective disorder. During this time (16 years), N4 has shown no symptom of DSM-III-R schizophrenia or related disorders. The determination of monozygosity was based on analysis of six independently assorting, highly heterogeneous repeat markers.

Genomic RDA

The RDA method used follows Lisitsyn *et al*[15] and Baldocchi and Flaherty[16] with minor modifications. One µg of genomic DNA was digested with 10 U of an appropriate restriction enzyme(s). Each RDA experiment dealt with matched pairs of genomic DNA to establish appropriate conditions. It started with ligation of appropriate restriction enzyme specific A adaptors (table 1) to the two digested genomic DNA samples. Subject specific amplicons were produced by PCR amplification of 100 ng of adaptor ligated genomic DNA. Amplicons designated as tester were prepared by digesting 5 µg with 20 U of the corresponding restriction enzyme to remove the A adaptors. This was followed by ligation to the appropriate B adaptor (table 1). The newly ligated adaptors on such a tester amplicon are expected to allow tester specific amplification only, following hybridisation with an excess (80:1) of driver amplicon lacking such adaptors.

Tester/driver hybridisation and selective amplification

For each hybridisation reaction, 40 µg of driver amplicon was mixed with 0.5 µg of B linked tester amplicon. The DNA was denatured at 98°C for five minutes and 1 µl of 5 mol/l NaCl was added, followed by hybridisation for 20 hours at 67°C. This produced the template for a PCR amplification (25 cycles) in the presence of primers specific to the B adaptor. The resulting PCR products are expected to represent sequences specific to the tester amplicon only, and were phenol/chloroform extracted and ethanol precipitated. They served as tester in the second round of the RDA by removing the B adaptors followed by ligation of C adaptors (table 1). When a third round of hybridisation was deemed necessary, the C adaptors were removed from the second round difference products and the B adaptors reused a second time. The amplified difference products of each round of RDA were examined on 6% polyacrylamide gels, bands cut from gels, cloned, sequenced, and used in database searches using the nucleotide Basic Local Alignment Search Tool (BLASTn) program. The results were confirmed by standard Southern blotting.

RESULTS

Testing principle of the method

In order to establish conditions for tester/driver hybridisation and PCR amplification of difference product(s), a set of control experiments was undertaken following Lisitsyn *et al*.[15] A human genomic DNA sample was divided into three aliquots of 10 µg each. The first was mixed with 150 pg of lambda DNA (pool A: estimated at one copy of lambda/human genome), the

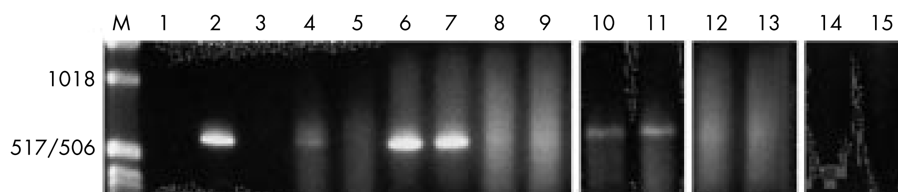

Figure 1 Results of PCR amplifications from RDA experiments involving introduced non-human bacteriophage lambda DNA as a target. Lane 1, negative control with no template. Lane 2 represents PCR product for lambda DNA cut with *Hin*dIII and linked to adaptor, amplified by adaptor specific primers. Lane 3 shows PCR product for a human DNA sample digested with *Hin*dIII without any adaptor (driver) and lanes 4 and 5 with appropriate adaptors (tester) as a template, amplified by adaptor specific primers. Lanes 6 and 7 are replicate experiments and represent one round of RDA using a tester containing 1000 copies of bacteriophage lambda genome per human genome and driver containing no lambda. Lanes 8 and 9 are replicate experiments and represent one round of RDA using a tester containing one copy of lambda per human genome and a driver containing no lambda. Lanes 10 and 11 show the second round results of these experiments. The band amplified in each case was confirmed by sequencing to represent the lambda target fragment. Lanes 12 and 13 are replicate experiments and represent one round of RDA using a tester and driver each containing one copy of lambda genome per human genome. Lanes 14 and 15 show the second round results of these experiments. Lane M is a 1 kb DNA marker from GibcoBRL.

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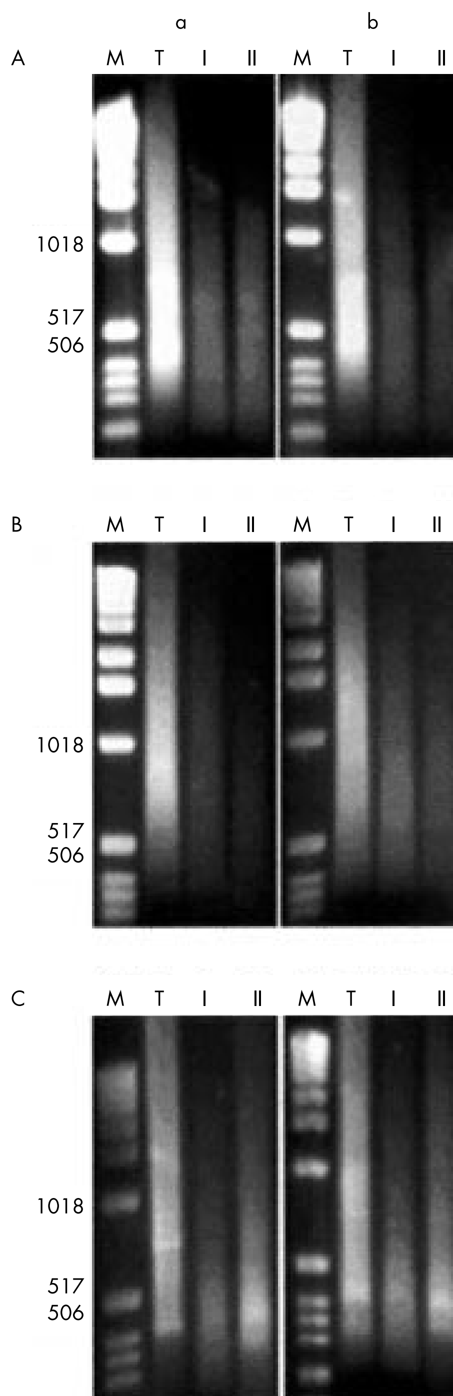

Figure 2 Results of two rounds of RDA using representations created by *HindIII* digestion (A), *Bam*HI digestion (B), and *Eco*RI digestion (C) on DNA extracted from a pair of MZ twins discordant for schizophrenia. Lanes labelled T are tester amplicon PCR amplified as a positive control. Lanes on the left hand side are RDA results using twin N3 (affected) as tester and twin N4 (unaffected) as driver. Lanes on the right hand side are RDA results using twin N4 as tester and twin N3 as driver. RDA rounds 1 and 2 in each case are denoted by I and II. There was no specific amplification of bands for any of the six RDA experiments taken to two rounds. Lanes labelled M contain a 1 kb DNA ladder (GibcoBRL).

second received 150 ng of lambda DNA (pool B: 1000 copies of lambda/human genome), and the third received no lambda DNA (pool C). Two rounds of RDA were performed in an attempt specifically to amplify a 570 bp lambda *Hin*dIII fragment. These experiments also included two sets of negative controls. The first involved pool C which contained no lambda and another where lambda was present in both tester and driver (fig 1). As a positive control, each tester amplicon (linked with A adapters) was PCR amplified resulting in an expected streak of amplification ranging from 300 bp to 1.5 kb (lanes 4 and 5). More importantly, one round of hybridisation was sufficient to amplify the lambda *Hin*dIII fragment from tester pool B (1000/human genome), while two rounds were needed to isolate the lambda *Hin*dIII fragment from tester pool A (1/human genome, lanes 10 and 11). The specific band (fig 1, lanes 10 and 11) was excised and cloned. The efficacy of the protocol was established by sequencing 12 randomly chosen clones. BLASTn database searches indicated that nine of the 12 clones matched the lambda *Hin*dIII fragment and was 99.8% identical to the 570 bp lambda fragment across nine clones. These results suggest that under these experimental conditions two rounds of RDA are required selectively to amplify difference products present at a single copy per genome in the tester. More importantly, these experimental conditions did not amplify such a sequence in a parallel RDA on a single genomic DNA used as tester as well as driver, with a single copy of lambda per human genome added to both (fig 1, lanes 14 and 15). The results establish that this RDA protocol should be efficient enough to identify a novel fragment, if present, between two genomic DNAs.

Application of RDA protocol to MZ twin samples

The RDA protocol developed above was applied to DNA extracted from the leucocytes of a pair of MZ twins discordant for schizophrenia using six different enzyme digest representations. These included *Hin*dIII, *Bam*HI, *Eco*RI, *Hpa*II, *Mbo*I, and a double digest involving *Hpa*II and *Mbo*I. Reciprocal hybridisations (N3 as tester/N4 as tester) resulted in a total of 12 RDA experiments, each carried to two or three rounds. It is apparent from fig 2 that the results of *Hin*dIII, *Bam*HI, and *Eco*RI representations (A, B, and C, respectively in fig 2) resulted in no selective amplification of difference products. Such results were obtained for each of the six reciprocal RDA experiments, suggesting that N3 and N4 do not differ with respect to the fragments generated by these enzymes. In order to test the pertinence of such a conclusion, additional experiments were undertaken involving three additional amplicons generated by *Hpa*II, *Mbo*I, and *Hpa*II plus *Mbo*I double digest. It is apparent from fig 3A that the three rounds of RDA did not yield difference product(s) in either reciprocal hybridisation of the *Hpa*II amplicons. The results were almost identical for two different conditions of $MgCl_2$ (2.5 mmol/l (1) and 4 mmol/l (2)) and suggest that there is no difference in *Hpa*II generated amplicons. Interestingly, the last two sets of amplicons generated by *Mbo*I and double digest *Mbo*I plus *Hpa*II did yield specific difference products shown in fig 3B and C, respectively, as described below.

RDA on *Mbo*I generated amplicons produced a distinct band at ~ 410 bp (designated fragment *M*R2L4T3D) which appears in round 2 at a lower $MgCl_2$ concentration with N4 amplicon as the tester. At a higher $MgCl_2$ concentration, however, this band is apparent in both hybridisation combinations. A third round refines this difference product with the ~410 bp band (designated *M*R3H4T3D) present in the lane containing N4 as tester only. Both bands were cloned, sequenced, and compared to the Genbank non-redundant (nr) database. The 410 bp DNA sequence of seven of the nine clones representing the round 2 fragment and six of the seven clones of the round 3 fragment matched (over 348 bp) to a human satellite III repeat sequence (central Sau 3A fragment of 1.177 kb EC repeat - gb[|]L12216.1[|]HUMSATIIX) with a score of ~561 (e-157). This conclusion was confirmed by probing a Southern blot containing *Hpa*II + *Mbo*I digested N3 and N4 genomic DNA hybridised with a random prime labelled *M*R3H4T3D probe (fig 4A). It yielded more than 15 bands common to both

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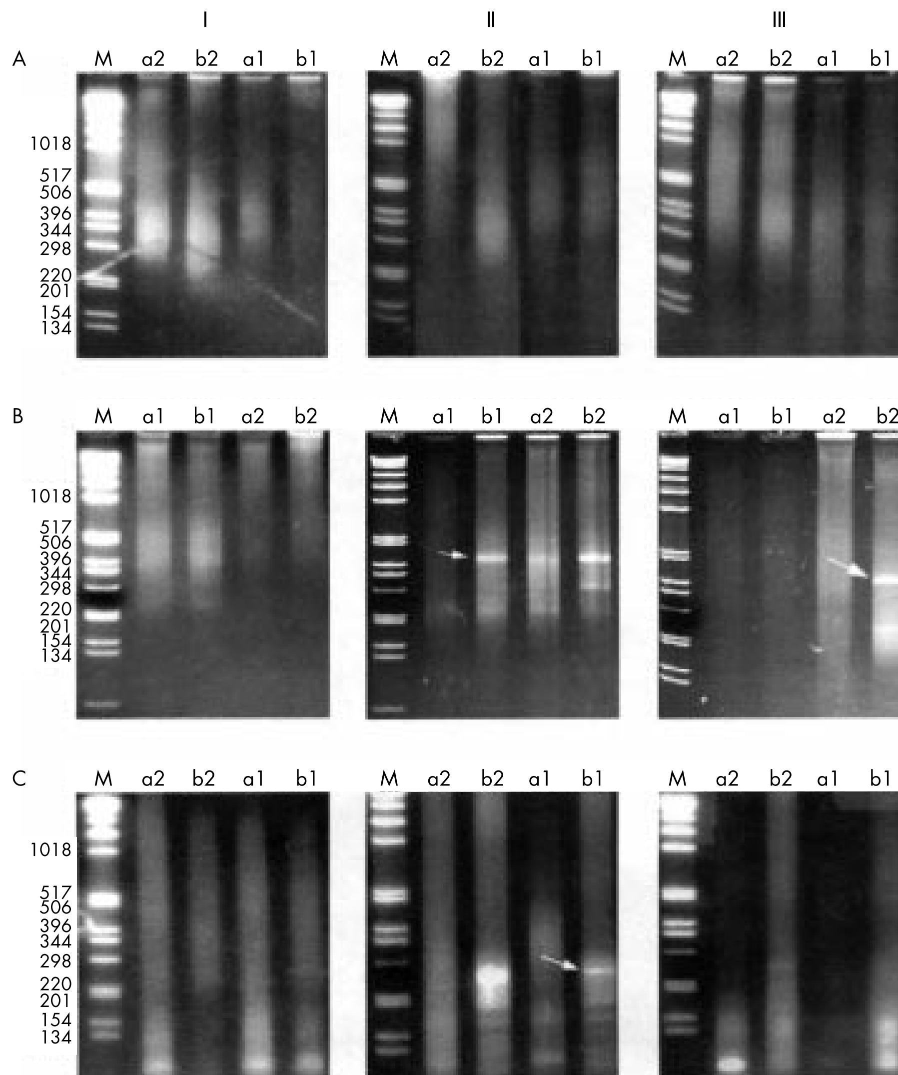

Figure 3 Results of RDA using representations created by *Hpa*II (A), *Mbo*I (B), and *Mbo*I plus *Hpa*II (C) digestion of DNA extracted from a pair of MZ twins discordant for schizophrenia completed to three rounds (I, II, and III) of RDA hybridisation. Lanes labelled (a) represent hybridisation involving N3 (affected) as tester and N4 (unaffected) as driver, while lanes labelled (b) have N4 as tester and N3 as the driver. The PCR for each hybridisation was carried out at two MgCl concentrations labelled as (1) (2.5 mmol/l) and (2) (4 mmol/l). Lane M in each gel represents the 1 kb DNA ladder (Gibco BRL). Bands identified by arrows were cut out, cloned, and sequenced. See text for details.

twins with no apparent difference between the samples, confirming that this sequence is present as a multicopy element repeated in every human genome. The presence of this fragment in reciprocal RDA hybridisations was considered to be because of copy number. The multicopy complexity of the sequence and the lack of Southern blot sensitivity to distinguish differences in copy number did not allow further experiments for establishing if the MZ twins are in fact different with respect to this sequence.

RDA analysis involving *Mbo*I + *Hpa*II generated amplicons with N4 as tester also produced a specific difference product at ~250 bp in round II, which did not persist as a prominent band in round III (fig 3C). The band from round II was excised, cloned, sequenced, and BLASTn compared to the GenBank database. Of five clones sequenced, a score of 400 (e-109) for four clones was achieved in a match to a *Pseudomonas aeruginosa* (bacterial) genomic fragment (gb|AE004949.1). Southern blot hybridisation with this fragment only produced specific banding in the lane containing amplicon from N4, implying that this fragment is not part of the human genome and was introduced during the amplification of N4 as possible contamination. Thus, the 250 bp difference product seen in RDA round II is proven to be a false positive.

DISCUSSION

Rare monozygotic twins who originate from a single fertilised egg, have been extensively used in the assessment of genetic *v* non-genetic determinants of complex diseases with the assumption that they are identical with respect to their DNA sequences. This critical assumption, however, has been questioned in more recent published reports,[2,4] a search of which shows that there are no comprehensive studies on genetic discordance involving MZ twins and no attempt has been made to assess the true DNA sequence identity of any pair of MZ twins. Given this lack of information, it is logical to question whether any MZ twin pair discordant for a common disease could be assumed to be genetically concordant, allowing their phenotypic discordance to be safely interpreted by non-genetic and epigenetic differences. A true answer will require exhaustive molecular sequencing of the twins, which is not practical.

This study was designed as an alternative to assess differences between leucocyte genomic DNA extracted from a pair of MZ twins discordant for schizophrenia using representational difference analysis.[15] Although this technique is extremely sensitive, it necessitates establishment of an effective protocol, using suitable controls. We used genomic DNA from a single person as the "tester" and as the "driver". By

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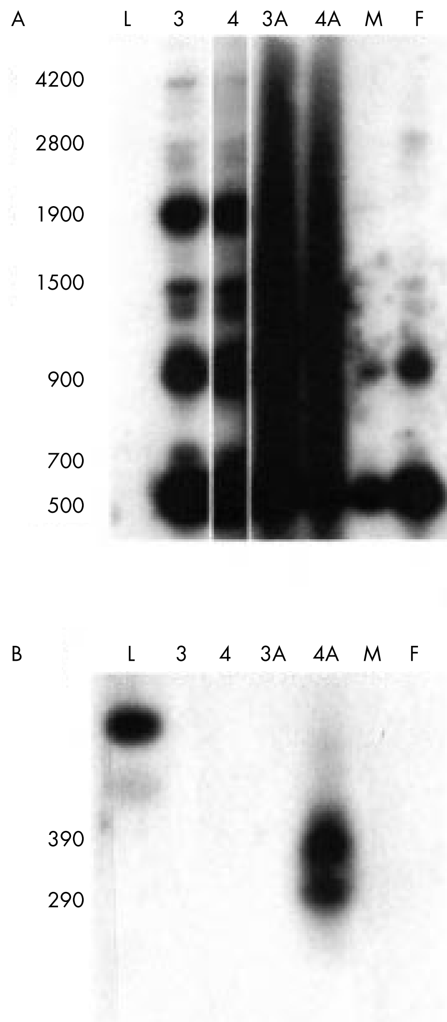

Figure 4 Southern blot hybridisation on twin N3 (3) and N4 (4) and control male (M) and female (F) genomic DNA digested with *Mbo*I plus *Hpa*II and the samples for the twins N3 and N4 (3A and 4A respectively). Panel A shows hybridisation of this blot with the band isolated from fig 3B and panel B shows hybridisation of the same blot with band (arrow) in fig 3C. Lane L represents the molecular marker lane.

introducing bacteriophage lambda genome into the "tester" pool at various concentrations, we created a genetic difference between the tester and driver representations as low as a single copy per human genome. The results of RDA on such tester and driver pools (fig 1) show that the protocol is effective in identifying a single copy difference between identical genomes and it should be effective in identifying any difference between MZ twins, if present.

The question of complete genetic concordance of MZ twins is not easy to resolve. This study, which deals with genomic RDA to identify differences in restriction fragments generated by six sets of restriction enzyme digestions (*Hin*dIII, *Eco*RI, *Bam*HI, *Hpa*II, *Mbo*I, and a double digest involving *Mbo*I plus *Hpa*II), probably comes close. The genomic DNA represented a pair of MZ twin discordant for schizophrenia, a common complex disease with an estimated heritability of ~80% and a MZ discordance of ~48%.[18] The enzymes were chosen for their ability to create different types of representations, with the expectation of covering different regions of the human genome. We have estimated that *Mbo*I representations may include 20%, *Hin*dIII representations 12%, *Eco*RI representations 8%, *Bam*HI and *Hpa*II representations each 1-2%, and the double digest with *Mbo*I + *Hpa*II representations 25% of the human genome.[15 19] A simple probability estimate suggests that in total the six different representations would cover >50% of the human genome. The results from 12 different RDA experiments show that the two members of this randomly selected MZ twin pair (N3, N4) do not differ over a major (>50%) fraction of the genome assessed. Such an extensive level of genome wide coverage for genetic discordance in any pair of MZ twins is unique, and argues that a genetic discordance between this MZ twin pair discordant for schizophrenia is not very likely.

It is apparent that little is known about DNA sequence differences between MZ twins, primarily because of the complexity of the genome and lack of simple, yet exhaustive and sensitive molecular technologies. Also, the available genome scanning approaches have rarely been used on MZ twins. One report[13] has used restriction landmark genome scanning (RLGS) and scanned a very small proportion of the genome, while another used amplicons specific to retroviral related sequences in RDA[11 20] to identify differences between MZ twins. These reports, however, are restrictive and do not offer an insight into genome wide differences between MZ twins. The results included in this study remain singular in attempting a comprehensive genome wide assessment of DNA sequence difference between MZ twins. Further, results of representations involving methylation sensitive restriction enzyme *Hpa*II suggest no methylation (epigenetic) differences between the discordant MZ twins studied.

There are two observations that offer an insight into the effectiveness of the protocol used. The first deals with the fragment identified in the RDA involving *Mbo*I representations (fig 3B). This sequence turned out to be an extensive repeat in the human genome (fig 4A), which could not be confirmed independently owing to complications of repeated sequences. The efficiency of a genomic RDA protocol, which relies on PCR to generate representations, may in fact be beyond the sensitivity level for heavily repeated sequences. Also, the twins may differ in the copy number for such multicopy sequences, but this could not be confirmed with the sensitivity of current Southern blotting technologies. The second observation deals with a fragment identified from the RDA representation generated by *Mbo*I plus *Hpa*II (fig 3C). Further assessment of this fragment using a Southern blot (fig 4B) has established that it is not part of the human genome. Rather, it was introduced during the generation of the representation by PCR in one of the two (N4) DNA samples. Once again, the protocol used has been effective in identifying it.

In conclusion, we have reliably assessed >50% of the genome of a pair of MZ twins discordant for schizophrenia using RDA and found no difference in the DNA sequence or *Hpa*II site methylation between them. It suggests that the discordance of MZ twins for common diseases including schizophrenia cannot be assumed to involve genomic discordance, without definite results, which may become practical with future genomic technologies.

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