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Research article

High-resolution physical map for chromosome 16q12.1-q13, the Blau syndrome locus

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Abstract

Background: The Blau syndrome (MIM 186580), an autosomal dominant granulomatous disease, was previously mapped to chromosome 16p12-q21. However, inconsistent physical maps of the region and consequently an unknown order of microsatellite markers, hampered us from further refining the genetic locus for the Blau syndrome. To address this problem, we constructed our own high-resolution physical map for the Blau susceptibility region.

Results: We generated a high-resolution physical map that provides more than 90% coverage of a refined Blau susceptibility region. The map consists of four contigs of sequence tagged site-based bacterial artificial chromosomes with a total of 124 bacterial artificial chromosomes, and spans approximately 7.5 Mbp; however, three gaps still exist in this map with sizes of 425, 530 and 375 kbp, respectively, estimated from radiation hybrid mapping.

Conclusions: Our high-resolution map will assist genetic studies of loci in the interval from D16S3080, near D16S409, and D16S408 (16q12.1 to 16q13).

Background

High-resolution genetic maps are essential to identifying and characterizing the gene(s) contributing to diseases, that is, they are essential for making the transition from positional cloning to the positional candidate approach. If the physical order of markers used for linkage analysis is incorrect, the position of the putative locus may at best be incorrect and at worst be found not to exist in the true interval. We were hampered in our efforts to fine map the gene for the Blau syndrome because we were faced with an

uncertain marker order in maps that had many inconsistencies.

The Blau syndrome (MIM 186580), an autosomal dominant granulomatous disease of childhood, is a rare familial granulomatous disease [1]. Affected individuals typically display one or more of the following granulomatous inflammations that have variable age-at-onset: acute anterior uveitis, arthritis, and skin rash. The phenotype of the Blau syndrome resembles another multisystem granu-

lomatous disorder, Crohn's disease (MIM 266600), and another autoimmune disease, psoriasis (MIM 177900). These three inflammatory diseases not only share phenotypic features, but a subset of putative risk loci mapped to an overlapping region on chromosome 16. Of the several putative susceptibility loci for Crohn's disease [2–7] and psoriasis [8], the Crohn's locus on 16q between markers D16S409 and D16S419 (56.1–65.6 cM from p-terminus) [5] and the psoriasis locus on 16q near D16S3110 [8] overlap with the locus for the Blau syndrome. Interestingly, another autoimmune disease ankylosing spondylitis (MIM 106300), also maps to chromosome 16q, however, it appears to map telomeric to the Blau syndrome interval [9,10].

While our studies were in progress, other groups detected mutations in the CARD15 gene for a subset of Crohn's disease patients, and in three small Blau syndrome families [11,12]. The lack of accurate maps is reflected in the position assigned to CARD15 in consecutive genome builds in that CARD15 should map to the 16q13 interval for Blau and Crohn's, yet in build 27 it was mapped close to the p-telomere of chromosome 16. In the current build, 29, it is located correctly (see additional data file 1, blau_map.pdf). Until the draft human genome is truly complete, detailed regional physical maps will be indispensable for genetic studies.

We have previously reported that the Blau syndrome susceptibility region resided between D16S298 and D16S408 [13]. Here we generated a high-resolution map extending from D16S3080, near D16S409, to D16S408 and placed four plausible candidate genes (CES1, MMP2, MMP15 and SCYA17) in the map. We also excluded the four candidate genes as the genes harbouring mutations causing the Blau syndrome.

Results

High-resolution RH map

To generate a framework order of markers, we used three RH panels (Table 1) and the RHMAP program package. We typed the GeneBridge 4 panel for 54 markers to verify that they occurred near, or in, the interval between D16S409 and D16S408. The G3 panel was typed for a total of 77 markers and the TNG panel for 83 markers. The markers were clustered into linkage groups by two-point analyses using RH2PT and a criterion of a lod score greater than or equal to 4. Multipoint analyses were performed on linkage groups as well as sparse linkage groups together with markers from other linkage groups. Several multipoint maximum likelihood framework maps were constructed using the RHMAXLIK program of the RHMAP package and equal retention probabilities and the branch ordering scheme options, which gives the highest probability of finding the correct order [14]. The G3 RH map

(see additional data file 1, blau_map.pdf) provided an order for the construction of the BAC contigs. Although the TNG panel generated a useful map, there were unresolved regions where it appeared that the panel did not provide sufficient data to differentiate between alternative marker orders. We merged the G3 and TNG data by establishing a G3 framework map that fixed the order of a subset of markers. Clusters of close markers containing at least two G3 mapped markers were then mapped relative to one-another using the TNG data. By integrating the G3 and the TNG maps, an overall contiguous RH map of the region between the markers D16S411 and D16S415 was constructed in which the marker order of most loci could be determined with a likelihood ratio of greater than 300:1 over the next most likely order (data not shown).

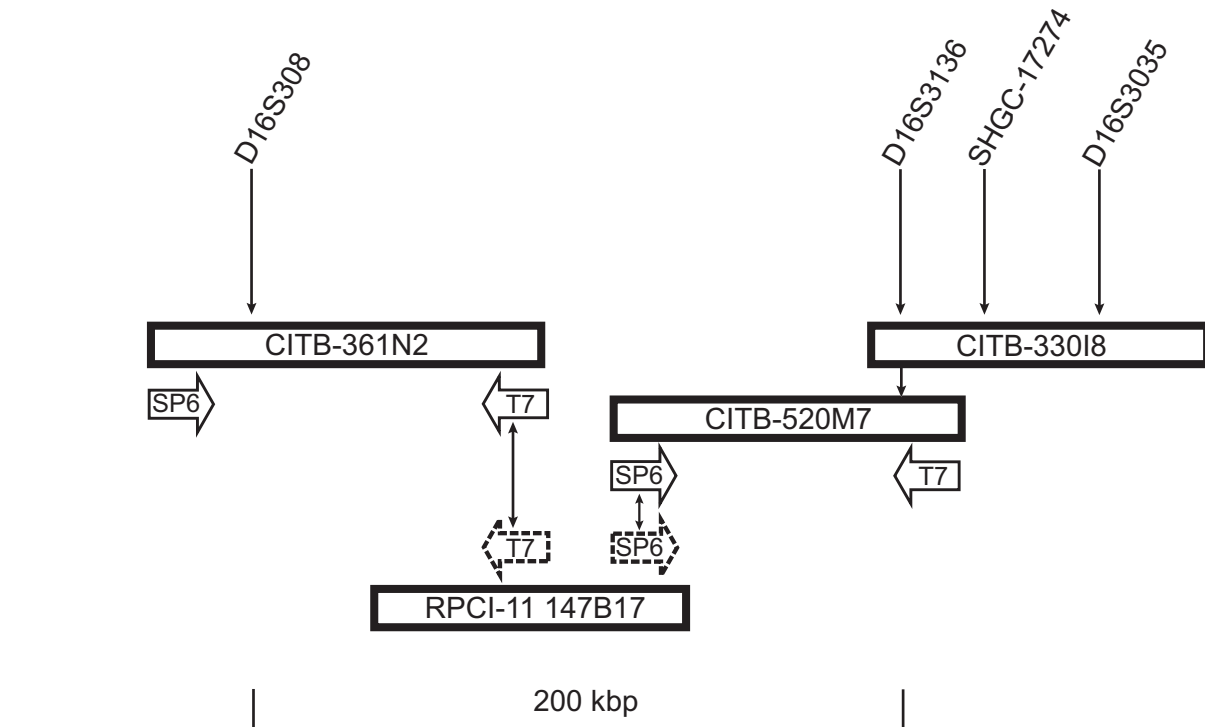
Table 1: Summary of radiation hybrid panel features.

	GeneBridge 4	Stanford G3	Stanford TNG
Resolution	Low	Medium	High
No. hybrids	93	83	90
Fraction of genome per hybrid	0.32	0.18	0.16
Average fragment size	10 Mbp	4 Mbp	800 kbp
X-ray dosage (cR)	3,000	10,000	50,000
Units	cR ₃₀₀₀	cR ₁₀₀₀₀	cR ₅₀₀₀₀
kbp per unit	270	25	4
Average resolution	1000 kbp	267 kbp	60 kbp

BAC contig construction

We carefully selected STSs from within or around the Blau candidate interval to screen two BAC libraries. More than 130 STSs were chosen from those in known genes, ESTs, polymorphic microsatellite markers, random genomic sequences, and BAC end sequences present in databases. Construction of a BAC clone contig was initiated by screening the CITB library with all the STS markers. The clone contigs were constructed based on the common BACs identified by two or more markers, using the RH map described above and the existing framework map resources as reference for ordering.

We initially identified 377 unique CITB clones with one or more STS hits from which a subset of 41 clones that contained at least two markers was used to construct 17 contig clusters (see additional data file 1, blau_map.pdf). These 41 non-redundant CITB clones in the contigs covered less than 50% of the broad Blau syndrome interval. The remaining 336 CITB clones were redundant in that they either were identified by a single marker or were identified by the same markers as the 41 non-redundant

**Figure 1**

Schema illustrating the use of BES alignment to close one of the 16 gaps in the early version of our map. T7 and SP6 in solid arrows indicate sequences derived from the T7 and SP6 promoters, respectively, of BAC clones. Dashed arrows indicate matches with sequences from the corresponding clone in the databases. Boxes represent clones and STS markers are indicated at top. An approximate distance derived from the G3 RH map is shown.

clones. Gaps in the contigs were closed by a combination of DNA sequence alignments of BESs with HTGS data, clone walking using RNA probes from end clones to hybridize to the RPCI-11 gridded filters, and verification of clone positions by PCR for STSs. From the BAC contigs, eight clones from both ends of the contig clusters were selected for sequencing using T7 and SP6 primers. Sequence alignments with these 16 end sequences revealed that two sequences, CITB_361N2-T7 and CITB_520M7-SP6, identified one common clone from the online HTGS, which was RPCI-11_147B17 (Figure 1).

BES alignment was a very feasible and practical technique for filling in the gaps with the availability of an ever-increasing number of BAC clone sequences in online databases. Using this approach, one gap between marker D16S2986 and Cda11d08 was closed. STS marker D16S2986 was initially used to search the HTGS data and one clone RPCI-11_357N13 was retrieved with 100%

identity. BES from clone 357N13 was then used to repeat the alignment searches and identified another clone RPCI-11_488J11, which overlapped STS markers Cda11d08 and WI-16741.

Two rounds of clone walking were carried out in order to either close the gaps or verify the order of the clone contigs. All the plasmid DNAs from these clones were isolated and completely digested by *Hind* III into linear fragments for run-off transcription. Since RNA probes were directly transcribed from human genomic sequence, it was necessary to test the probes for repetitive sequences. The presence of repetitive sequences can hinder the specific identification of BAC clones from the library filters. Of 44 probes tested, three probes produced multiple bands with the genomic DNA indicating that they contained repetitive sequences, and were, therefore, excluded from further analyses. We identified more than 400 clones from the RPCI-11 library of which 83 were incorporated (see addi-

tional data file 1, blau_map.pdf). After one round of clone walking, the physical map covered more than 80% of the broad Blau syndrome interval. A second round increased coverage to greater than 90%. Three unresolved gaps remain in the physical map (see additional data file 1, blau_map.pdf).

Comparison of BAC contig to other maps

We compared our map to the current, July 1, 2002, contigs generated by fingerprinting and available from the FPC database at Washington University Genome Sequencing Center. The FPC database has few clones from the CITB library and none that corresponded to the clones on our map (see additional data file 1, blau_map.pdf).

Of 83 RPCI-11 clones in our contigs, 63 were present in the FPC database; 1 was not placed on a contig, 6 were placed on contigs of different chromosomes, and 56 in three chromosome 16 contigs, ctg16020, ctg16021 and ctg16024. The gap between ctg16020 and ctg16021 corresponds to gap 1 (see additional data file 1, blau_map.pdf). FPC contig ctg16020 is oriented q-telomere to centromere, i.e. reversed with respect to our map. One clone, 420D3 was placed in ctg16020, however, our data suggest it should be in ctg16020 close to 122K22 and 444I18. Our data also reversed the order of clones 380E14, 210K18 and 535L14 in ctg16020. Aside from one minor difference with respect to clone 444I18 that our data place slightly closer to the q telomere, the remaining 37 clones of contig ctg16021 that were common to both maps agreed very well. There was a single clone in ctg16024, 463J22 that our data place in ctg16021, near 228I15. Interestingly, one of the clones, 327F22, that was placed on a contig from a different chromosome was the clone that contains all or part of CARD15.

The NCBI build current at the time our map was being finalized was build 25 (see additional data file 1, blau_map.pdf). There were a large number of discrepancies between our map and the build 25 map. The gene subsequently characterized as having mutations in Blau syndrome families was not in the interval. The discrepancies largely remained until builds 28 and 29. Build 29, the current build, of NCBI agrees very well with our map (see additional data file 1, blau_map.pdf).

Candidate genes

Four genes, CES1, MMP2, MMP15 and SCYA17, with relevant biochemical functions based on the literature and with map locations in the broad Blau susceptibility region as defined by our initial DNA linkage study for the Blau syndrome [13], were considered as candidates. CES1 is expressed in mature monocytes and macrophages, and is involved in the degradation of xenobiotics [15]. MMP2 or MMP15, or both, are connective tissue degrading enzymes

[16]. A hypothetical role for MMP2 and MMP15 in the Blau syndrome is that aberrant expression of MMP2 and MMP15 could inappropriately degrade connective tissue, which, in turn, could release stored cytokines and inflammatory mediators, such as tumor necrosis factor α and interleukins and potentially expose neo-epitopes. SCYA17 is a member of the CC group of chemokines that is involved in immunoregulatory and inflammatory processes [17–19]. It is a chemoattractant for T lymphocytes and is expressed constitutively in thymus and transiently in stimulated peripheral blood monocytes [20]. Interestingly, high levels of expression of SCYA17 were observed in lipopolysaccharide-stimulated human monocytes [21]. Sekiya et al. [22] showed that SCYA17 potentially played a role via a paracrine mechanism in the development of allergic respiratory diseases. Thus, inappropriate topical expression of SCYA17 could induce a localized immune or auto-immune response.

A total of 13 sequence variants were identified in the promoter region of the CES1 gene (Table 2) by sequencing 33 members from the Blau families. Seven thirteen-marker haplotypes could be assigned unambiguously (Table 3) using extended pedigree structures (data not shown), however, none of them was associated with the Blau syndrome phenotype. The CES1 gene was, therefore, excluded as a candidate for the Blau syndrome.

RH mapping of CES1 and MMP2 placed them close, and centromeric, to D16S408 (see additional data file 1, blau_map.pdf), whereas MMP15 and SCYA17 mapped telomeric to D16S408. The MMP15 locus was placed between D16S408 and D16S494 using the GeneBridge 4 RH panel. CES1 and MMP2 were mapped to clones in the BAC contigs (see additional data file 1, blau_map.pdf), whereas SCYA17 and the markers Cda1jb12 and R55777 were mapped to the BAC clones, CITB_387I12, CITB_437K19 and CITB_229H3, which map 1.1 Mbp telomeric to D16S408 according to the NCBI human genomic data (data not shown). Our own RH distance estimates also place these markers more than 1 Mbp telomeric to D16S408. In our G3 RH map CES1 was located outside a subsequently refined Blau syndrome interval that excluded several cM centromeric to D16S408, thus confirming the lack of association between CES1 haplotypes and the disease phenotype.

Mutations were characterized in the gene CARD15 for a few small families with the Blau syndrome [11,12]. We have characterized additional mutations in Blau syndrome families, however, only five of ten families had mutations in the exons and flanking sequences (X. Wang, H. Kuivaniemi, G. Bonavita, L. Mutkus, U. Mau, E. Blau, N. Inohara, G. Nunez, G. Tromp and C.J. Williams; in press). The gene was located in the approximate interval

Table 2: Summary of the sequence variants in the promoter region of the CES1 gene.

Sample	Gender	Pheno- type	Genotypes at locations												
			939	968	975	993	994	1012	1024	1028	1029	1032	1047	1081	1087
Blau-01	F	U	G/T	G/G	G/G	C/C	G/G	G/G	C/C	T/T	C/C	G/G	G/G	G/G	T/T
Blau-02	F	A	G/G	G/G	G/G	C/C	G/G	G/G	C/C	T/T	C/C	G/G	G/G	G/G	T/T
Blau-03	F	U	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-04	F	U	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	A/A
Blau-05	F	U	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-06	F	U	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-07	M	A	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	A/A
Blau-08	F	U	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-09	F	U	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-10	F	A	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-23	F	U	G/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-24	M	U	G/T	G/A	G/A	G/C	G/A	G/C	G/C	T/C	T/C	G/A	G/T	G/A	T/T
Blau-25	M	U	G/T	G/A	G/A	G/C	G/A	G/C	G/C	T/C	T/C	G/A	G/T	G/A	T/T
Blau-26	M	A	G/T	G/A	G/A	G/C	G/A	G/C	G/C	T/C	T/C	G/A	G/T	G/A	T/A
Blau-27	F	A	G/T	G/A	G/A	G/C	G/A	G/C	G/C	T/C	T/C	G/A	G/T	G/A	T/A
Blau-28	F	U	G/T	G/A	G/A	G/C	G/A	G/C	G/C	T/C	T/C	G/A	G/T	G/A	T/A
Blau-29	M	A	G/G	G/A	G/A	G/C	G/A	G/C	G/C	T/C	T/C	G/A	G/T	G/A	T/A
Blau-30	M	U	G/T	G/A	G/A	G/C	G/A	G/C	G/C	T/C	T/C	G/A	G/T	G/A	T/A
Blau-31	M	A	G/T	G/A	G/A	G/C	G/A	G/C	G/C	T/C	T/C	G/A	G/T	G/A	T/T
Blau-11	F	U	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/T
Blau-12	F	A	T/T	G/A	G/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/T
Blau-13	F	U	T/T	G/A	G/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/T
Blau-14	F	U	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/T
Blau-33	M	A	T/T	G/G	G/G	G/C	G/A	G/C	G/C	T/C	T/C	G/A	G/T	G/A	T/T
Blau-15	F	A	G/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-16	M	A	G/T	A/A	G/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-17	F	A	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/T
Blau-18	F	U	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/T
Blau-19	F	A	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/T
Blau-20	M	U	T/T	A/A	A/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/T
Blau-21	M	A	G/T	A/A	G/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-22	M	U	G/T	A/A	G/A	G/G	A/A	C/C	G/G	C/C	T/T	A/A	T/T	A/A	T/A
Blau-32	M	A	G/T	G/A	G/A	G/C	G/A	G/C	G/C	T/C	T/C	G/A	G/T	G/A	T/A

Genotypes were derived from five different families, members of one family are separated from those of other families by blank lines.

both cytologically and on the Santa Cruz map [http://genome.ucsc.edu/cgi-bin/hgGateway?db=hg10], but was located on chromosome 16p on the NCBI map (builds 25 to 28). We verified the location of CARD15 on our map by amplifying a 444 bp fragment of exon 4 from clone 327F22 in our map (see additional data file 1, blau_map.pdf).

Discussion

Correct marker order and intermarker distance are essential for linkage analysis and therefore require construction of accurate physical and genetic maps. Here we employed BAC contig and RH mapping to construct a high-resolution physical map, which spans approximately 7.5 Mbp of chromosome 16 between markers D16S3080 and D16S408. This map will definitely assist in the refinement

Table 3: Different haplotypes constructed from the sequence variants in the CES1 promoter region.

Sequence variant site	Haplotype						
	1	2	3	4	5	6	7
939	G	T	G	T	T	G	T
968	G	G	A	A	A	A	G
975	G	G	A	A	A	G	G
993	C	C	G	G	G	G	G
994	G	G	A	A	A	A	A
1012	G	G	C	C	C	C	C
1024	C	C	G	G	G	G	G
1028	T	T	C	C	C	C	C
1029	C	C	T	T	T	T	T
1032	G	G	A	A	A	A	A
1047	G	G	T	T	T	T	T
1081	G	G	A	A	A	A	A
1087	T	T	A	T	A	A	T

of the Blau syndrome locus as well as other loci mapping to this region, such as Crohn's disease [5], psoriasis [8] and possibly ankylosing spondylitis [9,10].

We constructed a high-resolution RH map (likelihood ratio of greater than 300:1 over the next likely order) with the medium-resolution G3 and high-resolution TNG whole-genome RH panels (Figure 1). This map allowed ordering of the polymorphic markers and estimation of the distances between them. The marker order provided the basis for construction of the BAC contig. The inter-marker distances estimated from RH data were substantiated by the BAC contig data when the average length of the BAC inserts was used to estimate intermarker distances. Our BAC contig provides more than 90% coverage of the entire Blau susceptibility region. The STS-based high-resolution physical contig consists of 124 BACs, and spans approximately 7.5 Mb as estimated from the number of BACs in the shortest tiling path and the average insert length of the BACs. We have used our map to refine the Blau syndrome interval (X. Wang, H. Kuivaniemi, G. Bonavita, L. Mutkus, U. Mau, E. Blau, N. Inohara, G. Nunez, G. Tromp and C.J. Williams; in press).

Another BAC contig map of chromosome 16q constructed by two-dimensional overlapping oligonucleotide (overgo) hybridization on high-density BAC filters has been published [23]. This map consists of 828 overgo markers and 3,363 BACs providing > 85% coverage of the long arm of chromosome 16. This contig map, however, does not cover the entire Blau syndrome region; it ends at the site of marker D16S2758 in our physical contig (see additional data file 1). The order for most of the markers is the

same on the two independently generated maps. Nevertheless, there are a few differences. For example, Han et al. [23] mapped the marker D16S2950 in the order of D16S2623, D16S2950 and D16S744, with the markers equidistant at approximately 130 kbp. We found the order to be D16S2623, D16S744 and D16S2950 with interval of approximately 130 kbp and 500 kbp, respectively. Also, the Han et al. [23] map has six regions in the Blau susceptibility interval which are either spanned with tenuous support or none at all. Two are real gaps with no overlapping clones and the other four are spanned by few clones that have either unusually long inserts or are missing some of the markers in the interval, or both.

Our contig also contains three gaps that we were unable to close by clone walking. One of them corresponds to one of the six gaps in the Han et al. [23] map. The other two are located in the region between markers D16S2758 and D16S408, which is not covered by Han et al. [23]. The common gap in both physical contigs indicates either a lack of STS markers in this region or an under-representation of clones even though two libraries were used in our study, or both. We estimate from RH data that the three gaps are only 425, 530, and 375 kbp in size and will not substantially affect DNA linkage analyses. The first gap (see additional data file 1), is also present in the FPC contigs, between ctg16020 and ctg16021, whereas the other two have been filled in.

Conclusions

We generated a high-resolution physical map from D16S3080, near D16S409, and D16S408 (16q12.1 to 16q13) because the correct marker order was required for further linkage studies with Blau syndrome families. A physical map was also necessary to locate, or verify the location of, candidate genes. Although there were substantial discrepancies as to the location of markers and genes at the time we initiated our mapping project, and even when this physical map was assembled into a final draft, the current NCBI map based on build 29, the FPC contigs, and the map produced here, agree remarkably well. The convergence of three independently constructed maps based on different approaches, suggests that the current maps are reliable, will perhaps undergo minor changes, and will be useful for mapping of disease loci to the interval.

Materials and Methods

PCR of STS markers

Primer pairs for most of the STS markers were purchased from Research Genetics, and some primers were synthesized by Integrated DNA Technologies, Inc. All test PCRs were carried out using 30 ng of template DNA with 1.5 µl GeneAmp 10 × PCR buffer II (Roche Molecular Systems, Inc.), either 1.5 mM or 3.0 mM Mg²⁺, 200 µM dNTPs, 0.2

μM each forward and reverse primer, and 0.375 units of AmpliTaq Gold Polymerase (Roche Molecular Systems, Inc.) in a 15 μl final volume. PCRs were performed in a model 9600 Thermocycler (Perkin-Elmer Cetus) using the following cycling protocol: initial denaturation at 94°C for 10 min; 94.5°C for 1 min, 55–60°C for 1 min, 72°C for 40 s for 35 cycles; followed by a final extension at 72°C for 3 min. PCR products were analyzed on 2% agarose gels.

Genotyping with polymorphic di-, tri-, or tetranucleotide-repeat markers was performed by PCR according to the conditions used in the test PCRs above except that one of the primers was ^{32}P -labeled with T4 polynucleotide kinase. Products were separated on 7% denaturing polyacrylamide gels with or without 50% formamide (Sequagel-6 or Accugel 19:1; National Diagnostics).

Construction of a RH map

The Whitehead Institute GeneBridge 4, and Stanford G3 and TNG whole genome RH panels (Research Genetics) were used to construct a RH map. Before typing the hybrid panels, the PCR conditions for STS markers were determined using hamster and human genomic DNA as control templates. PCR products were separated on agarose gels. PCRs with a band of appropriate size were considered positive and scored as 1, negative PCRs as 0 and ambiguous PCRs with bands of unexpected size as 2. The GeneBridge 4 RH map was constructed using a publicly available server at the Whitehead Institute at the Massachusetts Institute of Technology. The G3 and TNG maps were generated using the RHMAP 3.0 program package [14]. The RH2PT program was used initially to determine two-point lod scores and all the markers were divided into different linkage groups. Markers in different linkage groups were used to carry out maximum likelihood calculations to estimate breakage probabilities and distances between markers using another program RHMAXLIK. We established a G3 framework map of a subset of approximately evenly spaced markers. Additional markers were mapped in overlapping sets with G3 panel data until the likelihood ratio dropped below threshold. Clusters of closely spaced markers around framework markers were then mapped using the TNG data and RHMAXLIK. Map orders were verified by performing independent estimations of likelihood but including markers from adjacent clusters.

Screening of BAC libraries

A BAC library, CITB, which was generated at the California Institute of Technology in the form of DNA pools arranged in microtiter plates, was purchased (Research Genetics). This library offers approximately six-fold coverage of the whole genome with an average insert size of 130 kbp. The library is comprised of 147,456 distinct recombinants. For rapid screening, DNAs from the clones had

been pooled into 48 superpools, each representing eight 384-well plates that were pooled into eight plate pools, 16 row pools, and 24 column pools. PCR-based screening consisted of identifying positive superpools followed by screening of the appropriate plate, row and column pools. Typically, the three-dimensional screening identified one or more unique BAC clones from the 3,072 BAC clones in a superpool. Occasionally, multiple positives in a single plate (plate, row, column) resulted in non-unique positives (these also generated false-positives). PCRs were performed as described above except that 1 μl of pooled BAC library DNA was used as template and the annealing temperature for most markers was raised to 60°C to achieve greater specificity.

High-density filters of the Roswell Park Cancer Institute human RPCI-11 BAC clones were purchased from BACPAC Resource Center at the Children's Hospital Oakland Research Institute in Oakland, CA. Segments 1 and 2 of RPCI-11, with a total of 218,995 colonies corresponding to approximately twelve-fold genomic coverage, were used. The filters were screened iteratively by hybridization with pools of RNA probes prepared from the ends of BAC clones identified either by PCR-screening or prior rounds of hybridization. Hybridizations were performed using ULTRAhyb buffer (Ambion) and components of the Strip-EZ RNA kit (Ambion).

Prior to pooling, the probes were tested individually for the presence of repetitive elements by Southern hybridization to the parent BAC DNA and complete *Hind* III digests of human DNA. Probes that demonstrated a pattern consistent with the presence of repetitive elements were excluded.

Field-inversion gel electrophoresis (FIGE)

To determine the size of important BAC inserts, 2 μg of BAC DNA was first digested with 10 units of *Not* I (New England Biolabs). DNA fragments were then separated on 1% pulsed field certified agarose gel (Bio-Rad) in 0.5 \times GTBE buffer (0.5 \times TBE, 0.1 M glycine) by FIGE with a PC 750 Pulse Controller (Hoefer Scientific Instruments). The samples were prerun at 12°C and 8 V/cm for 1 h, and then run continuously for 17 h with forward pulse 1.2 s, reverse pulse 0.4 s and timing ramp 0.8/h. A Low-Range PFG Marker (0.13–194 kbp) (New England Biolabs) was used for estimation of BAC insert sizes. After electrophoresis, the gel was stained with ethidium bromide, visualized, and photographed with a GelPrint2000i system (BioPhotronics).

Verification of positive BAC clones

Screening for BAC clones, either by PCR of pooled DNA, or by hybridisation, produced a wide range of signal intensities. Although differences in signal intensity may be

considered as a measure of the evidence for the clones' positions in the contig, the variability of input target DNA made the conclusions unreliable. Positive clones were therefore rescreened by PCR with STS markers in the vicinity to eliminate false positive.

Positive clones were purchased from Research Genetics and colony-blot filters were prepared using a Biomek 1000 Automated Laboratory Workstation with a 96-pin replicator (Beckman Coulter). The gridded colony-blot filters were screened with individual RNA probes from the pools above. All positive clones were confirmed by PCR with appropriate primer pairs.

Preparation of RNA probes from the ends of BAC clones

BAC DNAs from standard alkaline lysis preparations [24] were completely digested with *Hind* III. RNA probes were synthesized from the ends of clones by run-off transcription using the Strip-EZ RNA kit (Ambion) and ³²P-UTP (ICN Radiochemicals). Unincorporated nucleotides were removed by size-exclusion chromatography on RNase-free Sephadex G-50 (Sigma) spin columns.

Candidate genes

Four genes, namely, CES1, MMP2, MMP15, and SCYA17, were selected based on their biochemical function and physical map locations prior to completion of our high-resolution map. DNA sequences for the four genes were obtained from GenBank (accession numbers for CES1: D21077; MMP2: U96098; MMP15: Z48482; SCYA17: AC004382). Primers to be used in PCR for each gene were designed using the Wisconsin Package Version 10.0 (Genetics Computer Group) and synthesized by Operon Technologies, Inc. PCR conditions other than annealing temperature used were the same as described above.

The physical locations of the four genes studied were verified by using RH panels as described below. Their locations were further refined by screening a BAC library, as discussed below, using gene-specific PCR primers. MMP15 and SCYA17 were located telomeric to D16S408 and thus outside the Blau syndrome interval and were excluded from further studies. MMP2 was placed outside of a refined Blau syndrome interval that excluded several cM centromeric from D16S408.

A 755 bp region, nucleotide position -660 to +95, of the CES1 gene was amplified by PCR from 33 members from five families with the Blau syndrome. PCR products were purified (GENECLEAN III, BIO 101 Inc.) to remove primers and other reagents, and sequenced (Thermo Sequenase[®], Amersham Life Science, Inc.). Haplotypes were derived from the inheritance of the variants in the families (families not shown). Sequencing products were separat-

ed on 7% glycerol-tolerant polyacrylamide sequencing gels (National Diagnostics).

To verify that the gene for CARD15 is located in the contig, a 444 bp genomic region, corresponding to nucleotide position 976 to 1227 of the CARD15 transcript (NM_022162.1) was amplified from BAC clones. Clone 327F22 yielded a band of appropriate size.

List of Abbreviations

BAC Bacterial artificial chromosome

BES BAC end sequence

CC group Group of chemokines with Cys-X-Cys motif

CES1 Carboxylesterase 1

CITB California Institute of Technology BAC

EST Expressed sequence tag

FIGE Field inversion gel electrophoresis

G3 Stanford G3 radiation hybrid panel

GTBE Glucose TBE

HTGS High-throughput genomic sequences

MMP Matrix metalloproteinase

MMP15 Matrix metalloproteinase 15

MMP2 Matrix metalloproteinase 2

NCBI National Center for Biotechnology Information

PEP Primer-extension preamplification

RH Radiation hybrid

RPCI Roswell Park Cancer Institute

SCYA17 Small inducible cytokine subfamily A (Cys-Cys), member 17

STS Sequence tagged site

TBE Tris Borate EDTA buffer

TNG Stanford TNG radiation hybrid panel

Authors' contributions

Author 1 (XW) performed typing and hybridisation experiments necessary to construct the RH map and BAC contig, participated in the writing and editing the manuscript; the work described here constituted a portion of his Ph.D. dissertation. Author 2 (HK) participated in the design and implementation of the study, contributed to writing and revising the manuscript. Author 3 (GB) performed some of the BAC DNA isolation and genotyping. Author 4 (CJW) participated in the design and implementation of the study, provided supervision of GB, and obtained funding for the study. Author 5 (GT) provided guidance to XW, participated in the design and implementation of the study, writing and editing the manuscript and obtained funding for the study.

Additional material

Additional files 1

Physical map of the Blau syndrome interval showing BAC clone contigs and markers. G3 RH indicates the map, generated from radiation hybrid data, which was used to estimate the size of each gap in the BAC contigs. NCBI 25 is the map based on NCBI build 25, the map from the NCBI that was current when the BAC contig was constructed and is shown at the top. NCBI 29 is the current map based on NCBI build 29. Horizontal arrowheads with "Cen" and "Tel" indicate the orientation of the centromere and q-telomere respectively. Transcripts, using HUGO (LocusLink) symbols of named loci are indicated below the scale line of build 29. Boxed symbols indicate that the genes were located on clones in this study, either to locate them or to verify their location. Horizontal lines above transcript symbols indicate approximate placement. For the BAC contig, horizontal lines are schematic representations of BAC clones and are not to scale; BACs are identified by text below each horizontal line. BAC clones are shown in clusters according to the library, CITB or RPC-11, from which they were isolated. Vertical bars indicate BAC clones that are positive for the corresponding markers indicated in vertical text above the BAC contigs. Two genes, matrix metalloproteinase 2 (MMP2) and carboxylesterase 1 (CES1) were mapped using markers developed here and are indicated as boxed markers. Caspase recruitment domain family member 15 (CARD15) was located to 327F22 using an exon 4-specific PCR and is also indicated as a boxed marker. A dotted line box around clones indicates the specific clones that differed between the FPC contigs and this map. Numbered vertical arrows on the bottom indicate the three gaps in the BAC contig.

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