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Report

Mapping of a Single Locus Capable of Complementing the Defective Heterochromatin Phenotype of Roberts Syndrome Cells

Lisa D. McDaniel,[1] Darrell J. Tomkins,[2,3,4] Eric J. Stanbridge,[5] Martin J. Somerville,[2,3] Errol C. Friedberg,[1] and Roger A. Schultz[1]

[1]Department of Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas; Departments of [2]Medical Genetics and [3]Pediatrics, University of Alberta, and [4]Darrell J. Tomkins Genetics Consulting, Edmonton, Alberta; and [5]Department of Microbiology and Molecular Genetics, School of Medicine, University of California–Irvine, Irvine

Roberts syndrome (RS) is a developmental disorder characterized by tetraphocomelia and a broad spectrum of additional clinical features. Most patients with RS exhibit characteristic cytogenetic phenotypes, which include an abnormal appearance of pericentromeric heterochromatin on metaphase chromosomes, referred to as "heterochromatic repulsion." In the present study, we use complementation of this abnormal cytogenetic phenotype as a means to identify a specific region of the normal human genome capable of rendering phenotypic correction. We screened the entire human genome, using a transient chromosome-transfer assay, and demonstrated complementation exclusively after the transfer of proximal chromosome 8p, a result subsequently confirmed by stable microcell-mediated chromosome transfer. Additionally, homozygosity mapping was used to refine the interval of this complementing locus to 8p21. The results are consistent with the notion that the single gene defect responsible for heterochromatic splaying and developmental abnormalities maps to chromosome 8p21.

In 1919, John Roberts described a rare developmental disorder, Roberts syndrome (RS [MIM 268300]) (Roberts 1919), that is characterized by tetraphocomelia (symmetrical limb reduction) and a broad spectrum of additional variably apparent characteristics that include prenatal and postnatal growth retardation, craniofacial abnormalities, cleft lip and palate, corneal clouding, prominent phallus, and cardiac and renal abnormalities (for a review, see the work of Van Den Berg and Francke [1993*a*]). This disorder has also been referred to as "pseudothalidomide syndrome," since infants born to women who took thalidomide during pregnancy had similar limb malformations (Jurenka 1976). Although RS and the SC phocomelia syndrome (MIM #269000) were originally described as distinct entities, they are now considered to be a single disorder with significant clinical heterogeneity (Waldenmaier et al. 1978; Tomkins et al. 1979; Fryns et al. 1980; Zergollern and Hitrec 1982).

RS appears to be represented by two genetic complementation groups, designated "RS+" and "RS−." Patients are classified into the two groups on the basis of their cytogenetic and cellular phenotypes. Cells and cell lines derived from patients with RS+ manifest a phenomenon evident in mitotic chromosomes that has been previously referred to as heterochromatic repulsion (HR), heterochromatic splaying (HS), or premature centromere separation (herein referred to as "HR") (Freeman et al. 1974; Louie and German 1981). Additionally, RS+ cells exhibit mild hypersensitivity to mitomycin C, cisplatin, γ radiation, and other DNA-damaging agents (Burns and Tomkins 1989; Van Den Berg and Francke 1993*b*), as well as cell-cycle abnormalities. Importantly, RS cell lines propagated in culture, including nontransformed fibroblasts, also exhibit random chromosome loss and gain, presumably the consequence of improper chromosome segregation (Jabs et al. 1989). Hereafter, the RS+ complementation group is designated as "RS."

Microcell-mediated chromosome transfer (MMCT) (McDaniel and Schultz 1992) is a technique whereby normal human chromosomes are transferred from donor to recipient cells via a microcell intermediate. Chromo-

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Address for correspondence and reprints: Dr. Lisa D. McDaniel, Department of Pathology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75390-9072. E-mail: lisa.mcdaniel@utsouthwestern.edu

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some-recipient clones are identified by selection for dominant markers and are analyzed for complementation of mutant recessive phenotypes (such as the HR associated with RS cells). However, an exhaustive analysis of all chromosomes for the identification of complementing loci by use of this approach would be very labor intensive. It would require the transfer of each tagged normal human chromosome, selection and long-term propagation of clonal isolates, and characterization of each of these clones for retention of the transferred chromosome and complementation of the relevant phenotype. Additionally, this strategy necessitates the use of immortalized cell lines representing the syndrome and maintenance of the cellular phenotype(s) of interest. Finally, with regard to the present study, initial experiments demonstrated that SV40 transformation of a fibroblast cell line (GM03913 [National Institute of General Medical Sciences Human Genetic Cell Repository]) of a patient with RS resulted in clones with an inconsistent HR phenotype, which compromised their utility in a complementation strategy (R.A.S. and L.D.M., unpublished observations).

To circumvent the aforementioned limitations, a transient whole-cell–fusion complementation assay was devised and was employed to introduce a limited number of human chromosomes from normal cells into RS cells, for rapid scoring for complementation of HR. The cell lines used were RS lymphoblastoid cell lines R20 and LB1 (Tomkins et al. 1979), Srsym1 (McDaniel et al. 2000), and Srsym2. The control donor cell line that was used, SV40-transformed VA-13 (American Type Culture Collection), was valuable because it contained both normal and rearranged copies of most chromosomes and was female in origin. Microcells were obtained from the donor cell line by treating cells in flasks with 25 μg/ml colcemid solution (Invitrogen) for 48 h, followed by centrifuging the flasks at 12,000 g for 1 h in the presence of 4 μg/ml cytochalasin B (Sigma). Microcells were collected and size selected by filtration and were fused with RS lymphoblastoid cells from patient cell lines, in the presence of 50% PEG 1500 (Roche), for 90 s. Ultimately, R20 proved to be the most efficient recipient, and all data reported are from fusions to that cell line. Complementation of a male cell line (R20) with a female donor afforded the opportunity to assess complemented metaphase cells for the presence of the Y chromosome as evidence that the metaphase originated from the R20 background. Fusion products were then grown for 48 h to allow complementation to occur in recipients that had received the complementing chromosome. Cells were grown for an additional 72 h, in the presence of hygromycin B, at a dose that did not significantly reduce the number of control metaphase cells but that lowered the overall number of R20 metaphase cells by 80%. This reduced the number of uncomplemented RS cells in the population. Surviving metaphase cells were then harvested. Chromosome-specific paints were used for FISH, to independently identify the copy number of each particular chromosome as well as to identify newly introduced rearrangements in both complemented and uncomplemented metaphase cells on the same slide. HR in RS cells is traditionally visualized by examination of fixed metaphase chromosomes stained by conventional C-banding techniques to highlight constitutive heterochromatin (Rooney and Czepulkowski 1986). In the present study, distamycin A (DA) with 4′-6-diamidino-2-phenylindole (DAPI) staining (Verma 1995) was employed to facilitate analysis of the HR effect in a fluorescence-based assay. DA/DAPI staining employed 50 μg/ml DA in PBS for 20 min. Slides were washed in PBS, stained with 0.5 μg/ml DA/DAPI for 15 min, mounted with Vectashield (Vector Laboratories). When DA and DAPI are used together, AT-rich areas of constitutive heterochromatin fluoresce in regions of chromosomes 1, 9, 15, 16, and distal Yq, which allows evaluation of HR with the simultaneous use of FISH to identify the chromosomes transferred.

Given that RS cells exhibit ongoing random chromosome loss and gain (Jabs et al. 1989), a numerical profile for each chromosome was generated for R20 parental cells by use of the same type of FISH studies employed to characterize the fusion products. Large numbers of complemented and uncomplemented metaphases were generated and analyzed on single FISH slides. Despite the aneuploid nature of the R20 cell line, our approach provided sufficient data to assess whether overrepresentation of a certain chromosome correlated with complementation. For nearly all chromosomes examined, the distribution of copy number for a given chromosome did not significantly vary among R20 parental, uncomplemented, and complemented metaphase cells (fig. 1*A*–1*C*). With use of this chromosome-painting approach, it was possible to rule out all autosomes, except chromosome 8 (fig. 1*D*). None of the other chromosomes displayed the increase in chromosome number in the complemented cells that was observed in chromosome 8. Consistent with this assignment, it was also observed that none of the complemented metaphases contained only one chromosome 8.

In addition to association of complementation with an increase in the numerical representation for a given normal chromosome, the SV40-transformed VA-13 cell line, used as the MMCT donor, offered structural rearrangements and deletions that could be identified in fusion products for correlation with complementation. BAC and YAC probes were localized to regions on the p and q arms of chromosome 8. These probes and chromosome 8 paints were then used to identify recurrent rearrangements in VA-13 metaphases. With use of these probes, it was noted that VA-13–derived 8p deletions

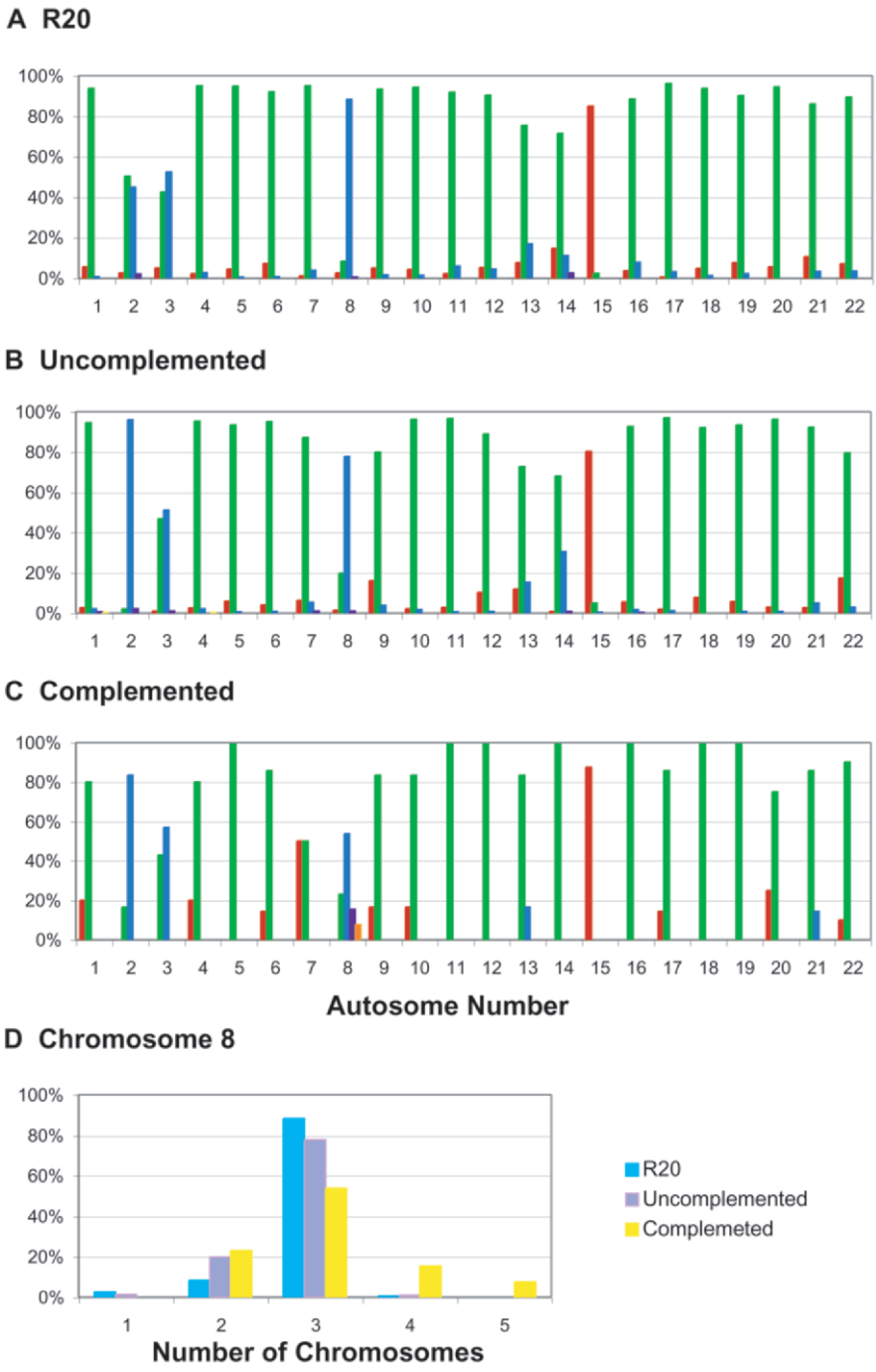

Figure 1 Profile of chromosome number in R20 parental cells, complemented cells, and uncomplemented cells after transient MMCT and FISH. *A*, R20 parental cells. With the use of one chromosome paint on each slide, 100–150 cells were characterized for chromosome number. *B*, Chromosome profile of uncomplemented metaphases after MMCT (150–500 metaphases scored). *C*, Chromosome profile of complemented metaphases on the same slide as the metaphases shown in panel B (10–30 metaphases scored). *A–C*, Bar colors indicate the percentage of metaphases with one (*red*), two (*green*), three (*blue*), four (*purple*), or five (*orange*) copies of each of the autosomes. *D*, Number of chromosome 8 sequences per metaphase in R20 parental, uncomplemented, and complemented metaphase cells in the same experiment. Chromosome 8 is the only chromosome that displayed an increased number in complemented cells.

failed to render correction (fig. 2*B*). Conversely, with retention of the p arm of chromosome 8, specifically with those metaphase cells that retained 8p12 sequences corresponding to YAC 847b12 and some distal material, results exhibited a 100% correlation with complementation. It was noted that the smallest complementing chromosome was positive for hybridization with YAC 847b12 but retained little additional distal material on the p arm of chromosome 8 (fig. 2*C*).

Collectively, the results of the transient complementation experiments are consistent with a map location of the complementing gene for RS in or near 8p12. These results predict that the stable transfer of chromosome 8 should correct the HR phenotype. To test this prediction, a mouse A9 monochromosomal hybrid bearing a human chromosome 8 that was "tagged" with a neomycin-resistance gene (neo^r) was used as a donor for MMCT to RS cells. GM03913 fibroblast cells were derived from the same patient as the R20 lymphoblastoid cells. As mentioned above, SV40-transformed GM03913 RS fibroblasts exhibited an unstable HR phenotype (R.A.S. and L.D.M., unpublished results). However, we established telomerase-immortalized GM03913 cells (reported elsewhere and herein as "TERT-RBS") (Ouellette et al. 2000) with a stable RS cellular phenotype, including HR, hygromycin B sensitivity, and random chromosome loss and gain. These cells were used as recipients for stable MMCT. Stable clones were recovered, and it was observed that one of eight clones was phenotypically complemented for HR (fig. 3*A*). In contrast, no complementation was seen after a neo-tagged chromosome 15 was introduced. These results confirm that chromosome 8 carries the complementing gene for RS. These complemented cells also exhibited resistance to hygromycin B, similar to that observed in control cells (data not shown).

Automated genotyping was used to evaluate sequences transferred from the chromosome 8 mouse/human hybrid MCH904.11 into recipient TERT-RBS cells. The seven uncomplemented clones and the single complemented clone were tested with 29 polymorphic markers spanning chromosome 8. Table 1 summarizes the allele sizes represented by the chromosome 8 sequences that are endogenous to the recipient cells and the one chromosome 8 that was provided by the donor hybrid. In this experiment, 27 of 29 markers were informative for the newly introduced chromosome 8 sequences. Analysis of the recovered clones revealed that the one complemented clone (clone 17) retained all the markers for the donated chromosome 8. Of the remaining clones, five of seven shared a common breakpoint in 8p11.2, and

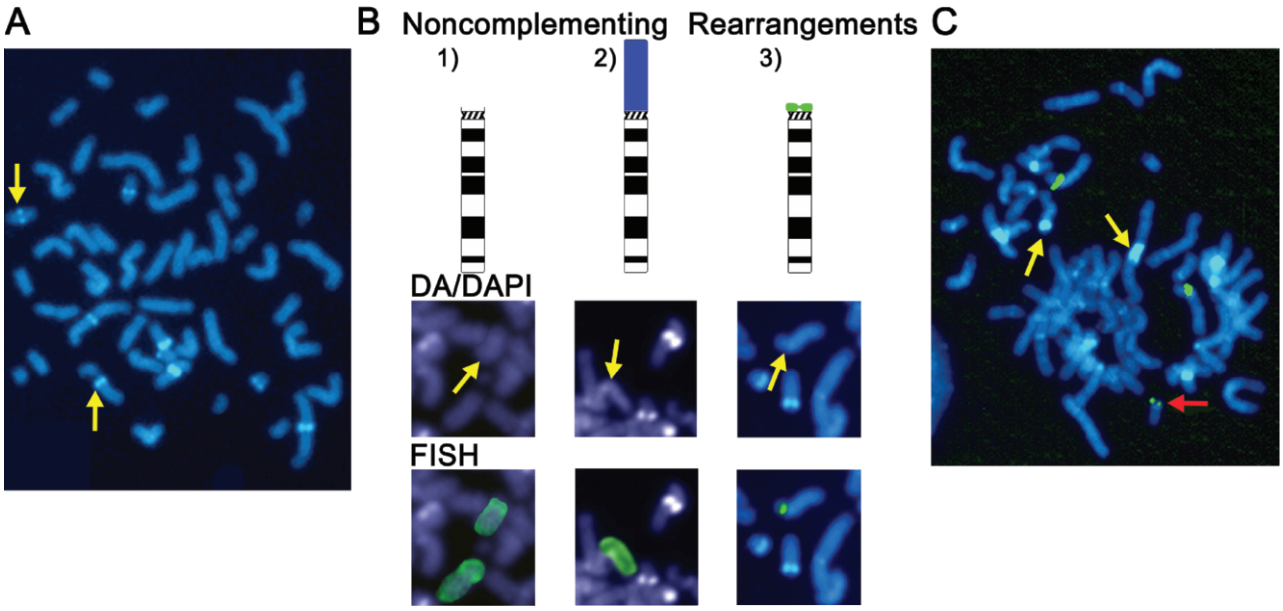

Figure 2 Characterization of metaphases observed by transient complementation. *A,* R20 metaphase, stained by DA/DAPI, displaying the HR phenotype in the constitutive heterochromatic regions of the autosomes. Yellow arrows indicate the HR phenotype in the heterochromatin of the chromosomes. *B,* Summary of the results for rearrangements that did not yield complementation of the HR phenotype, displayed by ideograms, DA/DAPI partial metaphases, and FISH. Yellow arrows indicate the uncomplementing transferred chromosome. Panels 1 and 2 show results with biotin-labeled chromosome 8 paint. Panel 3 shows results with biotin-labeled YAC 815d7 (8p11.21). All were detected with avidin–fluorescein isothiocyanate (FITC). *C,* Complemented metaphase, with the smallest fragment of chromosome 8 (indicated by the red arrow) detected by transient complementation assay probed with YAC 847b12 (8p12). Yellow arrows indicate the HR phenotype in the heterochromatin of the chromosomes.

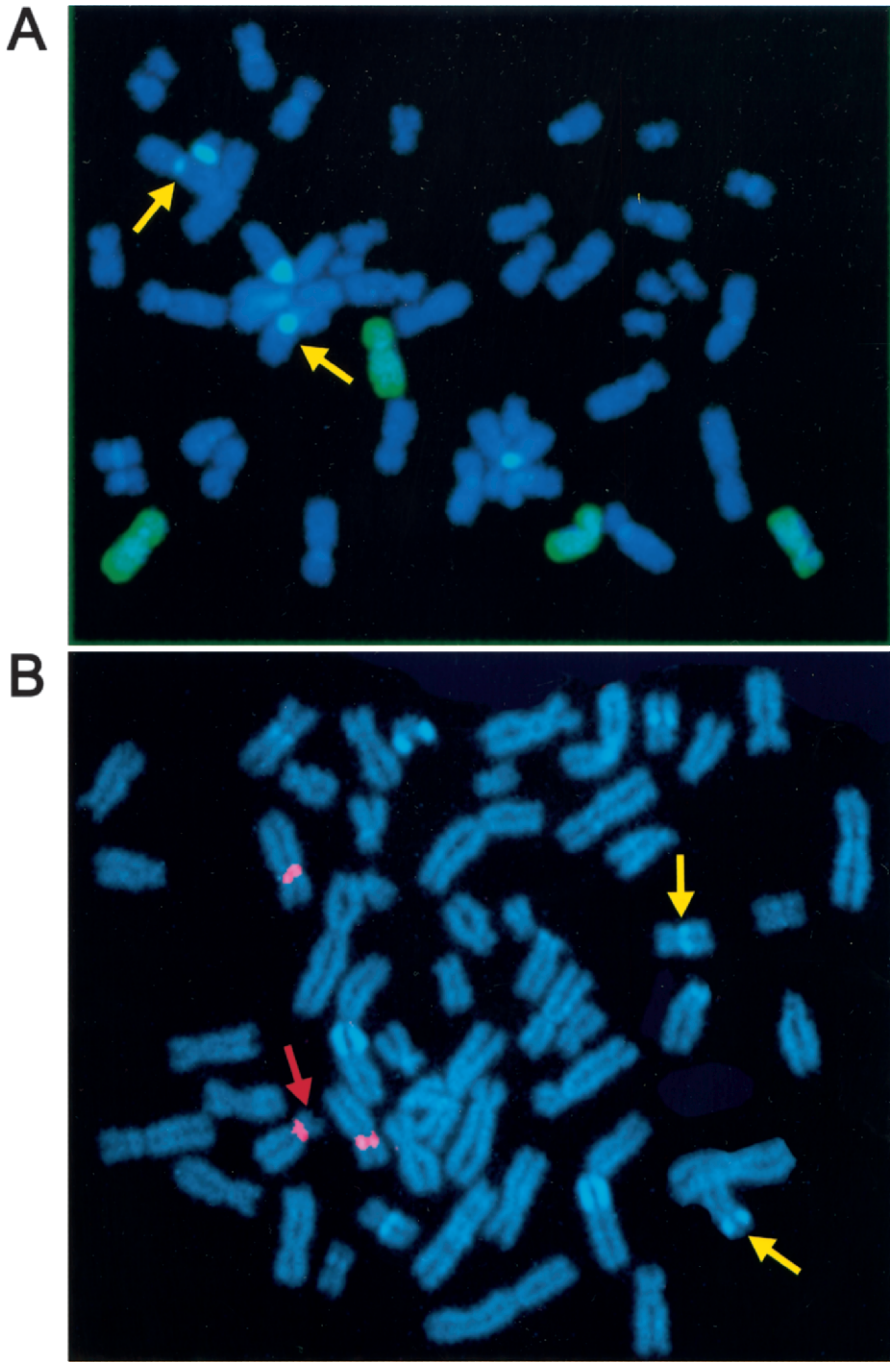

Figure 3 Stable introduction of chromosome 8 into TERT-RBS cells. Yellow arrows indicate the HR phenotype in the heterochromatin of the chromosomes in the DA/DAPI-stained metaphases. *A,* Metaphase exhibiting complementation of the HR phenotype in clone 17. Chromosome 8 paint, labeled with biotin and detected with avidin-FITC, reveals tetrasomy for chromosome 8 in the complemented metaphase, a result consistent with the fact that most of the TERT-RBS cells were trisomic for chromosome 8 prior to MMCT. *B,* The uncomplemented clone 18, probed using an chromosome 8 centromere probe directly labeled with Spectrum Orange. The red arrow indicates the contributed chromosome 8, with a significant portion of the p arm deleted.

Table 1

Allele Sizes in Parental Cell Lines and Clones from MMCT

	Allele Size(s) (bp) in									
			Clone							
Marker	MCH904.11	TERT-RBS	1	2	3	4	17	18	19	20
D8S264	155	151, 153	151, 153	151, 153	151, 153	151, 153	***151, 153, 155***	151, 153	151, 153	151, 153
D8S550	208	192, 194	192, 194	192, 194	192, 194	192, 194	***192, 194, 208***	192, 194	192, 194	192, 194
D8S1106	125	143	143	143	143	143	***134, 125***	143	143	143
D8S549	84	82	82	82	82	82	***82, 84***	82	82	82
D8S1145	270	273, 281	273, 281	273, 281	273, 281	273, 281	***270, 273, 281***	273, 281	273, 281	273, 281
D8S258	152	154, 156	154, 156	154, 156	154, 156	154, 156	***152, 154, 156***	154, 156	154, 156	154, 156
D8S282	265	267	267	267	267	267	***265, 267***	266	266	266
D8S1786	211	215	215	215	215	215	***215, 211***	215	215	215
D8S1752	156	145	145	145	145	145	***145, 156***	145	145	145
D8S1734	120	114	114	114	114	114	***114, 118***	114	114	114
D8S1725	266	268	268	268	268	268	***266, 268***	268	268	268
D8S1771	356	354	354	354	354	354	***354, 356***	354	354	354
D8S382	317	309	309	309	309	309	***309, 317***	309	309	309
D8S1048	204	204	204	204	204	204	204	204	204	204
D8S1839	175	175	175	175	175	175	175	175	175	175
D8S131	134	140	140	140	140	140	***134, 140***	140	140	140
D8S585	174	186	186	186	186	186	***174, 186***	186	186	186
D8S1820	111	119	119	119	119	119	***119, 117***	119	119	119
D8S1477	166	154	***154, 166***	***154, 166***	***154, 166***	***154, 166***	***154, 166***	***154, 166***	154	154
D8D505	115	113	***113, 115***	***113, 115***	***113, 115***	***113, 115***	***113, 115***	***113, 115***	113	113
D8S1121	118	115	***115, 118***	***115, 118***	***115, 118***	***115, 118***	***115, 118***	***115, 118***	115	115
D8S1821	142	165	***142, 165***	***142, 165***	***142, 165***	***142, 165***	***142, 165***	***142, 165***	142	142
D8S532	247	245	***245, 247***	***245, 247***	***245, 247***	***245, 247***	***245, 247***	***245, 247***	245	245
D8S1104	129	141	***129, 141***	***129, 141***	***129, 141***	***129, 141***	***129, 141***	***129, 141***	141	141
D8S1747	211	219	***219, 211***	***219, 211***	***219, 211***	***219, 211***	***219, 211***	***219, 211***	219	219
D8S1745	127	133	***127, 133***	***127, 133***	***127, 133***	***127, 133***	***127, 133***	***127, 133***	133	133
D8S1133	193	189	***189, 192***	***189, 192***	***189, 192***	***189, 192***	***189, 192***	***189, 192***	189	189
D8S1136	260	249	249	***249, 260***	249	249	***249, 260***	249	249	249
D8S1119	182	174	***174, 182***	***174, 182***	***174, 182***	***174, 182***	***174, 182***	***174, 182***	174	174

Note.—Numbers in bold italics represent cases in which the alleles from both R20 and the chromosome hybrid MCH904.11 were present in the clone. Markers that were uninformative are underlined.

two of seven had none of the alleles of the donated chromosome. FISH analysis confirmed the loss of distal 8p (fig. 3*B*). Note that the recovery of neo^r clones bearing this structure is consistent with a more proximal 8p location of the neo-tag in the chromosome 8 present in MCH904.11 hybrid cells (results documented by FISH [data not shown]). The loss of the p arm of chromosome 8 during the process of MMCT may be due to the clastogenic effects of cytochalasin B (Kolber et al. 1990) or to an event that may be stimulated by the amplification of the neo^r gene during selection.

We performed a genomewide scan for homozygosity. DNA from five unrelated patients with RS, patient S4134 and four previously mentioned patients (Tomkins et al. 1979), was amplified and analyzed for microsatellite markers. Chromosomes 1–11 were completed, and no loci were found to be homozygous in all patients, except on chromosome 8. On the basis of the above evidence for an RS complementing locus on 8p, we focused on this chromosomal arm to further refine the location of the complementing gene. In addition to known microsatellites, some SNPs and predicted variable repeats (Fondon et al. 1998) were used. The results presented in table 2 identify an interval of homozygosity, in all five patients with RS, mapping to 8p21, with no evidence of an alternative locus. Most patients exhibit homozygosity over a common 8.9-Mb interval; one patient, S4134, was homozygous for markers covering a 2.3-Mb region.

In summary, the results presented identify a single genomic interval on proximal chromosome 8p that is capable of complementing the HR phenotype and hygromycin sensitivity of RS+ cells. The results of homozygosity mapping confirmed the existence of an interval at 8p21 that is homozygous for all markers tested in five patients with RS. The results are consistent with the notion that the complementing locus for HR and the gene defect in patients with RS are one and the same and that it maps to chromosome 8p21.

Addendum.—While this manuscript was under review, *Nature Genetics* published "Roberts Syndrome Is Caused by Mutations in *ESCO2,* a Human Homolog of Yeast *ECO1* That Is Essential for the Establishment of

Table 2

Homozygosity Mapping of Patients

Marker	Location[a] (Mb)	Results of Mapping for Affected Patients Who Were: Unrelated R20	Unrelated Srsym1	Unrelated Srsym2	From an Isolated Community R23/LB1	From an Isolated Community S4134	Band
D8S264	2.1	HT	HT	HT	HT	HT	8p23.3
D8S1099	6.1	HT	HM	HT	HT	HM	8p23.2
D8S277	6.5	HT	HM	HM	HM	HM	8p23.1
D8S1469	9.1	HT	HT	HT	HT	HT	8p23.1
D8S550	10.9	HT	HM	HM	HT	HT	8p23.1
D8S1130	11.9	HT	HT	HT	HT	HT	8p23.1
D8S1106	12.9	HM	HT	HM	HT	HT	8p22
D8S511	14.7	HM	HT	HT	HM	HT	8p22
D8S549	15.7	HM	HM	HT	HM	HM	8p22
D8S1145	18.4	HT	HM	HT	HT	HT	8p22
D8S258	20.4	HT	HM	HT	HT	HM	8p21.3
D8S282	21.4	HM	HM	HM	HT	HT	8p21.3
D8S1116	21.4	HM	HM	HT	HT	HT	8p21.3
D8S136	22.5	HM	HM	HT	HT	HT	8p21.3
D8S1786	22.5	HM	HM	HM	HT	HT	8p21.3
D8S1733	22.6	HM	HM	HM	HT	HM	8p21.3
D8S1752	22.7	HM	HM	HT	HM	HT	8p21.3
D8S1734	22.9	HM	HM	HT	HT	HT	8p21.3
AF279339[b]	23.3	HM	HM	HT	HM	Not done	8p21.3
AC044891[b]	24.2	HM	HM	HM	HM	Not done	8p21.2
AC024958[b]	24.4	HM	HM	HM	HM	Not done	8p21.2
D8S1989	24.7	HM	HM	HM	HM	HT	8p21.2
D8S1725	25.1	HM	HM	HM	HM	HT	8p21.2
D8S1771	25.5	HM	HM	HM	HM	HM	8p21.2
DOCK5ca10[b]	25.5	HM	HM	HM	HM	Not done	8p21.2
D8S382	26.3	HM	HM	HM	HM	HM	8p21.2
PPP2R2A[c]	26.3	HM	HM	HM	HM	Not done	8p21.2
D8S1048	26.0	HM	HM	HM	HM	HM	8p21.2
PTK2B[d]	27.3	HM	HM	HM	HM	HM	8p21.2
Exon 26[e]	27.4	HM	HM	HM	HM	HM	8p21.2
D8S131[f]	27.4	HM	HM	HM	HM	HM	8p21.2
D8S1839	27.4	HM	HM	HM	HM	HT	8p21.1
SCARA3-F1[b]	27.5	HM	HM	HM	HM	Not done	8p21.1
D8S585	27.6	HM	HM	HM	HM	HT	8p21.1
SCARA3-F2[b]	28.0	HM	HM	HM	HM	HM	8p21.1
D8S1820	28.0	HM	HM	HM	HM	HT	8p21.1
D8S1477	32.2	HM	HM	HT	HT	HT	8p12
D8S505	34.6	HM	HM	HT	HT	HT	8p12
D8S1121	35.9	HM	HM	HT	HT	HT	8p12
D8S1747	37.1	HM	HM	HM	HT	HT	8p12
D8S1821	38.5	HT	HT	HT	HM	HT	8p12
D8S255	40.0	HM	HM	HT	HT	HT	8p11.21
D8S1104	40.7	HM	HM	HT	HT	HM	8p11.21
D8S532	40.9	HM	HM	HT	HT	HT	8p11.21

Note.—HM = homozygous; HT = heterozygous.

[a] Map locations are based on the University of California–Santa Cruz Genome Browser.

[b] Variable-repeat regions were predicted from the sequence by POPPOUS (Fondon et al. 1998).

[c] SNP *rs3824232*: 51 bp before exon 10 splice site (allele frequency unknown).

[d] SNP *rs1045512*: exon 2, p.Lys54Lys, Srsym1, Srsym2, and LB1 = A (allele frequency 0.775), R20 = G (allele frequency 0.225).

[e] SNP *rs751019*: p.Thr838Lys, R20, and LB1 = C (allele frequency 0.453); Srsym1 and Srsym2 = A (allele frequency 0.547).

[f] *D8S131*: all patients are homozygous for the most common allele (allele frequency 0.791).

Sister Chromatid Cohesion" (Vega et al. 2005). That publication describes homozygosity mapping and identification of the *RBS1* gene called "*ESCO2*." *ESCO2* resides within the 8.9-Mb interval on chromosome 8p21, as defined in the present study, which confirms that the disease-causing gene and the locus that can complement the HR phenotype are one and the same.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (RS and SC phocomelia syndrome)
University of California–Santa Cruz Genome Bioinformatics, http://genome.cse.ucsc.edu/

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