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Genes for Two Autosomal Recessive Forms of Chronic Granulomatous Disease Assigned to 1q25 (*NCF2*) and 7q11.23 (*NCF1*)

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Summary

Chronic granulomatous disease (CGD) is a heterogeneous group of inherited disorders of impaired superoxide production in phagocytes. The most common X-linked recessive form involves the *CYBB* locus in band Xp21.1 that encodes the membrane-bound beta subunit of the cytochrome b_{558} complex. Two autosomal recessive forms of CGD result from defects in cytosolic components of the phagocyte NADPH oxidase system, $p47^{phox}$ (NCF1) and $p67^{phox}$ (NCF2). By using human cDNA probes we have mapped the genes for these proteins to chromosomal sites. The combined data from Southern analysis of somatic cell hybrid lines and chromosomal in situ hybridization localize *NCF1* to 7q11.23 and *NCF2* to band 1q25. The *NCF1* localization corrects an erroneous preliminary assignment to chromosome 10. In the mouse, the locus corresponding to *NCF2* (*Ncf-2*) was mapped with somatic cell hybrid panels and recombinant inbred strains to mouse chromosome 1 near *Xmv-21* within a region of conserved homology with human chromosome 1 region q21-q32. A second site, probably a processed pseudogene, was identified on mouse chromosome 13.

Introduction

Chronic granulomatous disease (CGD) refers to a group of rare inherited disorders, characterized by recurrent severe bacterial or fungal infections. Affected patients' phagocytes are unable to kill ingested bacteria by generating microbicidal superoxide from oxygen (respiratory burst), and this defect leads to the formation of granuloma tissue (Klebanoff and Clark 1978). Different components of the defective membrane-bound NADPH-oxidase system are absent or nonfunctional in the genetically heterogeneous forms of CGD. The X-linked recessive form (X-CGD), present in about two-thirds of all cases, involves a phagocyte-specific cytochrome b (Segal et al. 1983), a heterodimeric glycoprotein with tightly associated subunits of 91,000 and 22,000 molecular weight (Parkos et al. 1987). The X-CGD gene (*CYBB*), mapped to band Xp21.1 (Francke 1984; Francke et al. 1985), was cloned on the basis of its location and tissue-specific expression (Royer-Pokora et al. 1986) and was subsequently shown to encode the larger subunit of the cytochrome b_{558} complex (Dinauer et al. 1987; Teahan et al. 1987). All patients with X-CGD, those with the common cytochrome b–negative form and an unusual family with cytochrome b present, who have been studied at the molecular level to date were found to have mutations at the *CYBB* locus (Royer-Pokora et al. 1986; Dinauer et al. 1989*b*; Francke et al. 1990). The smaller 22-kilodalton (kD) subunit gene has also been cloned and found to be expressed in many cell types (Parkos et al. 1988). Mutations in this gene are likely to be responsible for the rare autosomal recessive cytochrome b–negative form of CGD (Weening et al. 1985; Orkin 1989; Parkos et

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al. 1989). However, in both forms of cytochrome b–negative CGD, X-linked as well as autosomal, both subunits are absent, suggesting that stable expression of one requires expression of the other subunit gene (Parkos et al. 1989).

The functional defect in the autosomal recessive cytochrome b–positive forms of the disease (AR-CGD) is the inability of the cytosol of phagocytes to activate the membrane components of the NADPH oxidase system in response to stimulation. Molecular defects for two distinct forms of AR-CGD have been identified (Nunoi et al. 1988; Volpp et al. 1988; Bolscher et al. 1989; Clark et al. 1989; Curnutte et al. 1989). Approximately 90% of AR-CGD patients lack an M_r = 47,000 protein (called $p47^{phox}$, or NCF-1) of the cytosol oxidase component. The cDNA for this protein has been cloned and sequenced, and its product has been used to restore the defective neutrophil function in cytosolic extracts of patients in this group (Lomax et al. 1989; Volpp et al. 1989). A less common form of AR-CGD is due to a defect in the M_r = 67,000 cytosolic factor ($p67^{phox}$, or NCF-2) (Clark et al. 1989). The cDNA for this factor encodes a 526-amino-acid protein that restores oxidase activity in phagocyte cytosol extracts from patients with p67 deficiency but not from those with p47 factor defects (Leto et al. 1990). A sequence motif common to both neutrophil cytosol factors is also present in the noncatalytic domain of *src*-related protein kinases, phospholipase C, nonerythroid α-spectrin, *ras*-p21 GTPase–activating protein (GAP), and myosin I of dictyostelium (Leto et al. 1990). The functional role of the conserved sequence motif may involve interaction with common GTP-binding proteins or components of the cytoskeleton. We have determined the chromosomal position of the two NCF genes in human, and of Ncf-2 also in mouse, to gain further insight into the evolutionary relationships of these protein genes and to map two loci for a human genetic disorder.

Material and Methods

The same cloned probes were used for in situ hybridization and Southern analysis. Probe NCF1–8A is a 1.33-kb *Eco*RI fragment that comprises the human nearly full-length cDNA of the p47 neutrophil cytosolic factor (Lomax et al. 1989). Probe NCF2-10 is a 2.16-kb *Eco*RI fragment that contains the almost complete human cDNA of the p67 cytosolic factor (Leto et al. 1990). For Southern analysis, the probes were ^{32}P-labeled by random priming, were hybridized to filters of DNA from human, mouse, rat, Chinese hamster, interspecies cell hybrids, and recombinant inbred strains of mice that had been digested with various restriction enzymes, as described elsewhere (Hsieh et al. 1989*b*).

The origin and characterization of our mapping panels with defined subsets of human and murine chromosomes have been summarized elsewhere (Francke et al. 1986; Hsieh et al. 1989*b*). A panel of 11 Chinese hamster × human hybrid cell lines was constructed from five different series of hybrids in such a way as to allow gene assignments to be made to any human autosome and the X chromosome, with exclusion of all other chromosomes by at least two discordant hybrids. For regional localizations of genes on individual human chromosomes, panels have been derived that contain hybrids with defined overlapping regions in the absence of an intact copy of the respective chromosome. The composition of the regional mapping panel for chromosome 1 has been described elsewhere (Hsieh et al. 1990).

For mapping in mouse, a panel of 12 Chinese hamster × mouse hybrids that collectively contain all mouse chromosomes except for number 11 is complemented by a rat × mouse hybrid cell line (RTM9) in which a Rb(11;13) fusion product is the only mouse chromosome present (Joyner et al. 1985; Münke et al. 1986). Hybrid cell lines were expanded in culture and harvested simultaneously for DNA extraction and chromosome analysis. DNA from the AK×D strains of recombinant inbred mice and from five inbred strains that are progenitors of RI strains was obtained from the Jackson Laboratory (Bar Harbor, ME).

For in situ hybridization, the probes were labeled by nick-translation and hybridized to prometaphase spreads with a normal 46,XX chromosome content according to a method described elsewhere (Francke et al. 1986). The association of silver grains with chromosome bands was determined on simultaneously G-banded spreads in which all chromosomes were identifiable.

Results

Mapping of Human NCF1 *Gene*

In *Bam*HI-digested human DNA, five fragments—8.6, 5.7, 4.8, 2.1, and 1.4 kb in size—hybridized with the NCF1 cDNA probe (fig. 1, lane 2). All human fragments were separable from weakly cross-hybridizing Chinese hamster fragments (fig. 1, lane 1). In the hybrid panel, the five human fragments were concordant

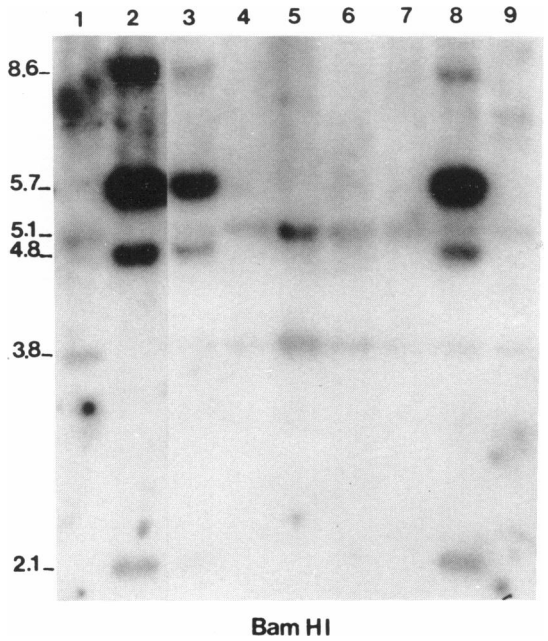


Figure 1 Southern filter of *Bam*HI-digested DNA from Chinese hamster (lane 1), human control (lane 2), and Chinese hamster × human hybrid cell lines (lanes 3–8) hybridized with labeled NCF1–8A probe. Only the hybrids in lanes 3 and 8 are positive for all human fragments, including a 1.4-kb fragment not shown.

with each other and with chromosome 7 (fig. 1). The two positive hybrids are shown in lanes 3 and 8 of figure 1. In addition, one of the two hybrids excluded from the discordancy analysis in figure 1 had chromosome 7 at low frequency and generated a weak 5.7-kb signal at the position of the strongest hybridizing band (not shown). The other excluded hybrid contained only the short arm of chromosome 7 and was negative for the human fragments. All other chromosomes were excluded from carrying NCF1-related sequences by two or more discordant hybrids, with the exception of chromosome 4, which had only one discordancy (table 1). Parts of chromosome 4 were further excluded by hybrids containing rearranged chromosomes 4 (hybrids that are not included in table 1).

After in situ hybridization, 39 randomly selected chromosome spreads were found to have 275 labeled sites (i.e., approximately seven sites/per cell). A site on the proximal long arm of chromosome 7 at subband 7q11.23 was frequently labeled on both homologues, often with a cluster of silver grains or with a distinct grain over each chromatid (fig. 2*A*). Thirty cells (77%) had label at this site on one or both chromosomes 7. Of the 78 specific sites available in the 39 cells, 43 (55%) carried label, and these represented 16% (43/275) of all labeled sites. The remaining silver grains were distributed randomly, and no other chromosomal region was labeled above background (fig. 2*B*).

We conclude that the human genome contains a single site for the *NCF1* gene, encoding the p47-phox neutrophil cytosolic factor, that is located in band 7q11.23. This result corrects erroneous preliminary data, assigning *NCF1* to chromosome 10, that were previously reported in an abstract (Hsieh et al. 1989*a*).

Mapping of Human NCF2 *Gene*

The human cDNA probe hybridized to six *Bgl*II fragments, of which three (10.0, 4.1, and 2.8 kb) were consistently scorable in hybrid cell DNA, as they were clearly separated from the major Chinese hamster fragments at 9.3, 6.0, and 4.6 kb (fig. 3). Fragments in the 2.4-kb size range were shared by both species. In

Table 1

Human NCF1 Sequences and Human Chromosomes in Hamster × Human Somatic Cell Hybrids

	Human Chromosome																						
Hybridization/Chromosome	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
+/+	0	0	1	1	0	1	2	0	1	1	1	1	1	1	2	0	0	1	1	1	1	1	2
−/−	6	7	4	6	6	4	7	5	7	8	4	6	5	2	4	5	7	3	5	6	3	3	1
+/−	2	2	1	0	2	1	0	2	1	1	1	1	1	1	0	2	2	1	1	1	1	0	0
−/+	3	2	4	1	2	5	0	4	2	1	3	3	4	5	5	3	2	6	4	3	5	6	2
Total no of discordant hybrids	5	4	5	1	4	6	0	6	3	2	4	4	5	6	5	5	4	7	5	4	6	6	2
Total no. of informative hybrids	11	11	10	8	10	11	9	11	11	11	9	11	11	9	11	10	11	11	11	11	10	10	5

Note.—Data are numbers of hybrids that are concordant (+/+ or −/−) and discordant (+/− or −/+) with the human NCF1 fragment. Hybrids in which a particular chromosome was structurally rearranged or present in fewer than 10% of cells were excluded.

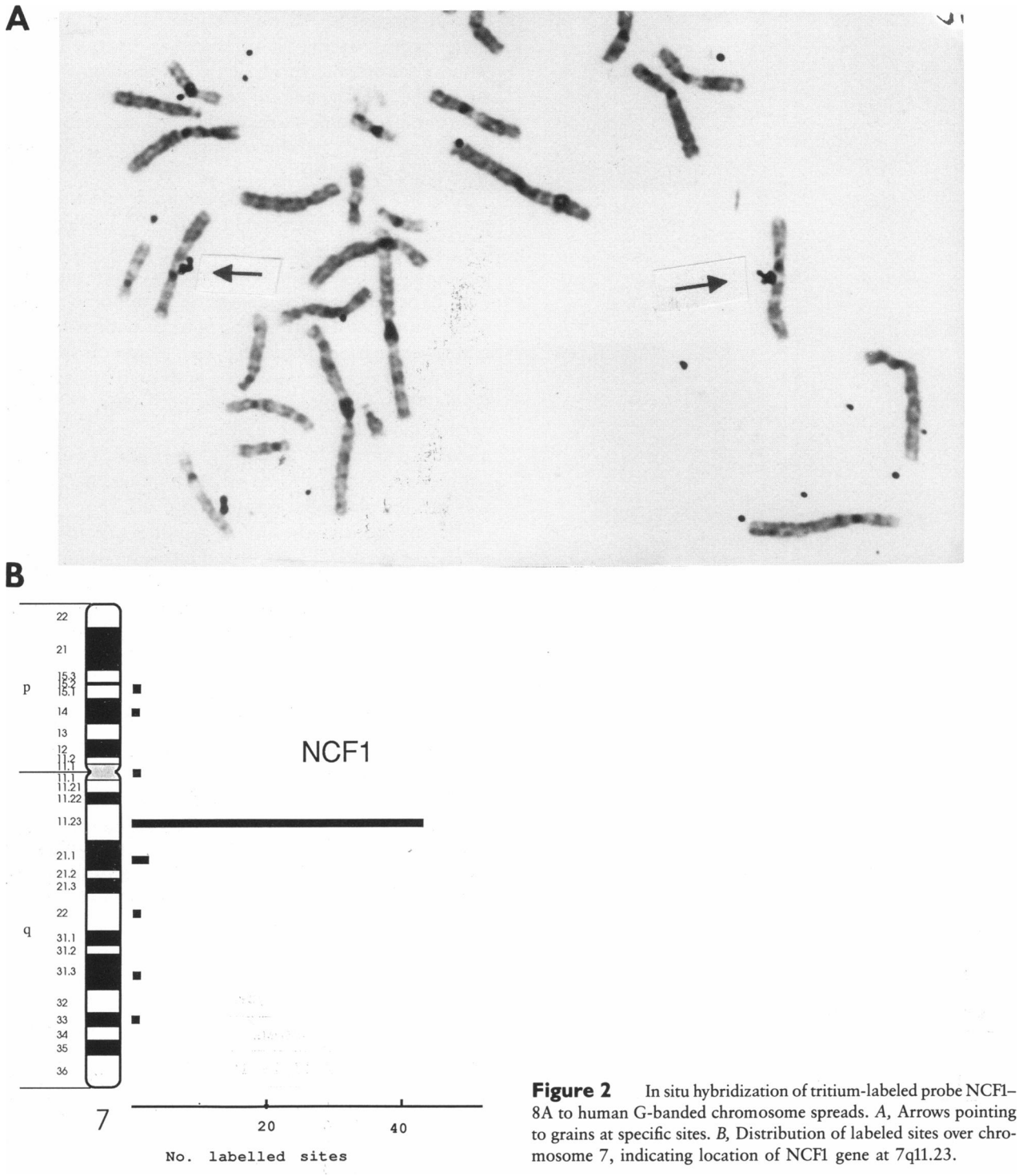


Figure 2 In situ hybridization of tritium-labeled probe NCF1–8A to human G-banded chromosome spreads. *A*, Arrows pointing to grains at specific sites. *B*, Distribution of labeled sites over chromosome 7, indicating location of NCF1 gene at 7q11.23.

the hybrid panel, the human fragments were concordant only with chromosome 1 (table 2). Analysis of a regional mapping panel assigned *NCF2* to the long arm, region cen-q32 (fig. 4*A*, *left*).

When 33 random metaphase spreads were scored for silver grains after in situ hybridization with human NCF2 cDNA probe, a total of 180 labeled sites were recorded (5.5/cell). A single site of specific hybridiza-

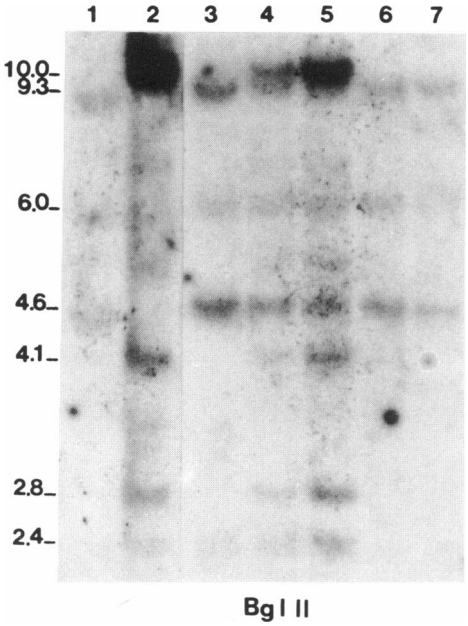


Figure 3 Filter hybridization of ^{32}P-labeled NCF2-10 probe to *Bgl*II-digested DNA from Chinese hamster (lane 1), human (lane 2), and Chinese hamster × human hybrid cell lines (lanes 3–7). Hybrids in lanes 4 and 5 contain all human fragments, while hybrids in lanes 3, 6, and 7 contain only Chinese hamster fragments.

tion was present at band 1q25, with minor grain scatter from 1q23-q32 (fig. 4*A*, *right*). Thirty-one (94%) of the cells had label at this site, usually on both chromosomes 1 (fig. 4*B*). Of all available specific sites, 64% (42/66) were labeled, and these represented 23% (42/180) of all labeled sites scored. No other chromosomal site was labeled above background. The data are consistent with the conclusion that the human genome contains a single site for NCF2 sequences, at 1q25.

Mapping of Ncf-2 *in Mouse*

While mapping of the mouse gene for NCF1 was not successful, because of insufficient cross-hybridization of the human cDNA probe, the human NCF2 probe revealed four to six fragments in mouse DNA cleaved with commonly used six-base cutters. These fragments, however, did not all segregate together in hybrid cell panels. For *Bgl*II (fig. *5a*) and *Hin*dIII (not shown), a single fragment that was also the most strongly hybridizing of all fragments was concordant with chromosome 13 (3.6-kb *Bgl*II and 6.0-kb *Hin*dIII fragments), while the remaining bands cosegregated with chromosome 1 (table 3).

RFLPs found with *Eco*RI, *Pst*I, *Bam*HI, *Bgl*II, and *Hin*cII distinguished the AKR/J pattern from that seen in C3H/HeJ, C57BL/6J, C57L/J, and DBA/2J strains. The polymorphism involved bands mapped to chromosome 1, not the one mapped to 13. When 23 AK×D recombinant inbred strains were typed for the *Bgl*II polymorphism shown in figure *5b*, the *Ncf-2* strain distribution pattern was identical to that reported for the endogenous xenotropic virus-21 locus *Xmv-21* (Frankel et al. 1989) (table 4). The 95% confidence limits for the recombination frequency (r) between *Xmv-21* and *Ncf-2* is 0–.0476. *Xmv-21* has been mapped to mouse chromosome 1 by linkage to markers in the *Pep3-to-Ly-22* region that is known to be homologous to human chromosome region 1q21-q32, which contains the *NCF2* locus.

Discussion

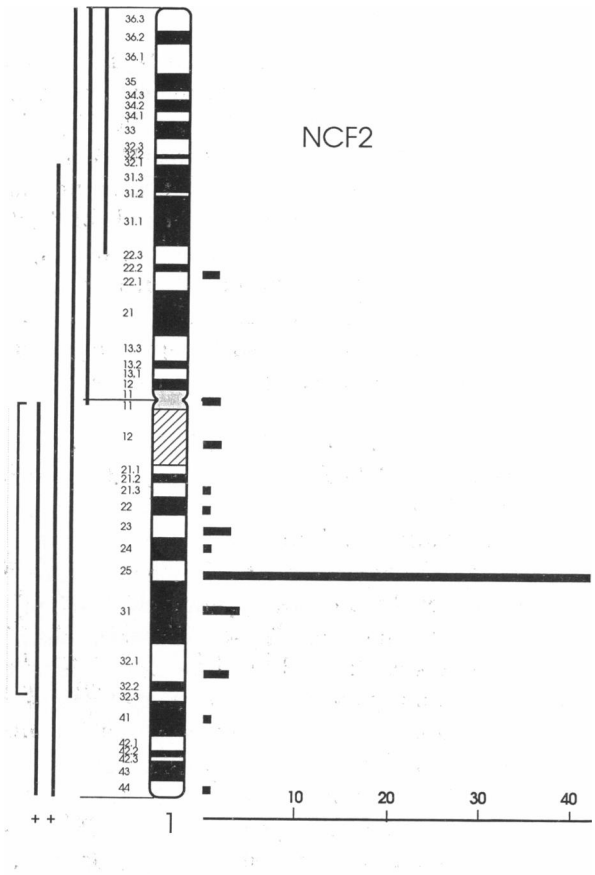
The biochemically and genetically heterogeneous group of CGDs caused by inability of phagocytes to generate superoxide can now be classified by mutant

Table 2

Human NCF2 Fragments and Human Chromosomes in Rodent × Human Somatic Cell Hybrids

	Human Chromosome																						
Hybridization/Chromosome	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
+/+	3	1	1	1	0	2	0	2	1	0	0	2	1	2	2	2	1	2	3	1	2	3	1
−/−	8	6	3	5	5	4	5	6	6	6	4	6	4	2	3	6	6	2	6	5	4	3	1
+/−	0	2	2	1	3	1	2	1	1	3	1	1	2	1	1	1	2	1	0	2	0	0	0
−/+	0	1	4	1	2	4	2	2	2	2	4	2	4	4	5	1	1	5	2	3	4	4	3
Total no. of discordant hybrids	0	3	6	2	5	5	4	3	3	5	5	3	6	5	6	2	3	6	2	5	4	4	3
Total no. of informative hybrids	11	10	10	8	10	11	9	11	10	11	9	11	11	9	11	10	10	10	11	11	10	10	5

Note.—Data are numbers of hybrids that are concordant (+/+ or −/−) and discordant (+/− or −/+) with the human NCF2 sequence. Hybrids in which a particular chromosome was structurally rearranged or present in fewer than 10% of cells were excluded.



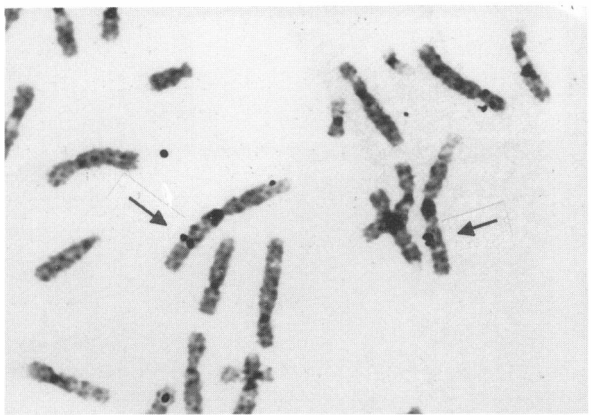


Figure 4 Regional mapping of *NCF2* on chromosome 1. *A, left,* Regions of chromosome 1 present in somatic cell hybrid lines (vertical bars), correlated with presence (+) or absence (−) of human-specific restriction fragments, that assign *NCF2* gene to region cen-q32 (*bracket*). *A, right,* Distribution of labeled sites over chromosome 1 in 33 cells after in situ hybridization of NCF2–10 probe and autoradiography indicating location of *NCF2* gene at 1q25. The horizontal axis is the number of labeled sites. *B,* Photomicrograph of labeled partial human metaphase spread. Arrows point to labeled specific sites at 1q25.

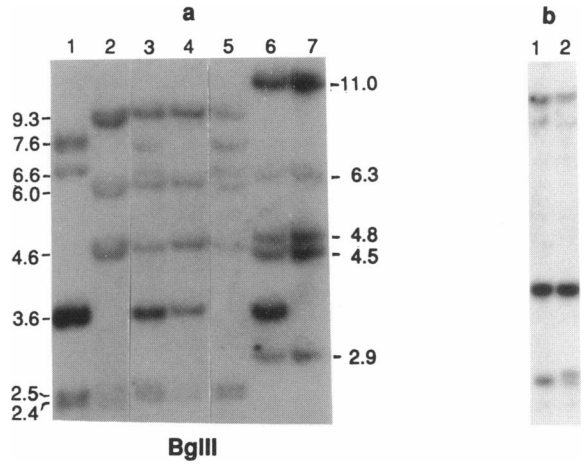


Figure 5 *A,* Somatic cell hybrid mapping of *Ncf-2* in mouse. Lane 1, Mouse control. Lane 2, Chinese hamster control. Lane 7, Rat control. Of the three Chinese hamster × mouse hybrids shown (lanes 3–5), that in lane 3 is positive for all mouse-specific fragments, and that in lane 5 is positive only for the 7.6-, 6.6-, and 2.5-kb fragments. The hybrid in lane 4 and the rat × mouse hybrid in lane 6 are positive only for the 3.6-kb fragment. *B,* RFLP used for recombinant inbred strain mapping of *Ncf-2* involving the 2.5/2.4-kb *Bgl*II fragments. Lane 1, AKR. Lane 2, DBA pattern.

gene loci (table 5). The genes for four components of the defective phagocyte NADPH oxidase system have been cloned, sequenced, and mapped. Although in vitro reconstitution of a functional oxidase from the products of the four cloned genes has not yet been accomplished, there is biochemical evidence that at least one additional cytosolic factor is necessary (Nunoi et al. 1988). However, there is no strong clinical evidence for a form of CGD involving a defect in a protein other than those coded for by the four cloned genes. In a classification based on biochemical parameters, at least six different types of CGD have been proposed (Curnutte 1988). Some of them are represented by a single or very few individuals. Molecular genetic studies are needed to determine whether one of the four known CGD genes is involved in these rare cases. For example, in a family with the rare cytochrome b–positive X-CGD, a single base change in the CYBB sequence was detected (Dinauer et al. 1989*b*), and the mutant phenotype was shown to cosegregate with an *Nsi*I RFLP detected by the CYBB cDNA (Francke et al. 1990). Thus the mutation in this family involves the same gene for the β subunit of cytochrome b that is mutated or deleted in the common X-linked cytochrome b–negative form.

The heterogeneity in onset and clinical severity, even

Table 3

Relationship of Mouse-specific *Ncf-2* Sequences with Mouse Chromosomes in Rodent × Mouse Somatic Cell Hybrids

Hybridization (*Bgl*II 7.6, 6.6-kb)/Chromosome	Mouse Chromosome																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X
+ / +	8	8	5	3	2	4	7	4	2	5	0	6	2	3	7	4	6	4	6	7
− / −	4	2	3	3	3	4	3	3	3	5	4	3	3	4	2	2	2	4	2	3
+ / −	0	1	4	5	6	2	1	4	6	3	9	3	5	5	2	4	3	4	3	1
− / +	0	3	2	2	1	1	2	2	1	0	1	2	2	1	3	3	3	1	2	2
Total no. of discordant hybrids	0	4	6	7	7	3	3	6	7	3	10	5	7	6	5	7	6	5	5	3
Total no. of informative hybrids	12	14	14	13	12	11	13	13	12	13	14	14	12	13	14	13	14	13	13	13
Total no. of hybrids discordant for *Bgl*II 3.6 kb	7	7	5	5	9	5	5	6	3	4	5	4	0	7	6	6	7	4	6	8

Note.—Data are numbers of hybrids that are concordant (+ / + or − / −) and discordant (+ / − or − / +) with the mouse *Ncf-2* sequences. Hybrids in which a particular chromosome was structurally rearranged or present in fewer than 10% of cells were excluded.

among members of the same family, suggests that there may be other factors that interact with the oxidase system. Variation in NADPH metabolism could be a contributing factor. For example, deficiency in glucose-6-phosphate dehydrogenase or in glutathione peroxidase can cause reduction in oxidative burst activity. Furthermore, both cytochrome b subunits and the p47 cytosolic factor are phosphorylated on stimulation of phagocytes (Caldwell et al. 1988; Okamura et al. 1988; Segal 1988). Protein kinase C and other kinases may be involved (Kramer et al. 1988).

The predicted amino acid sequences of NCF1 and NCF2 share limited similarity with both region A of *src* and related proteins, GAP, gap-*crk*, and nonerythroid α-spectrin. Genes for these proteins are scattered over different chromosomes (Leto et al. 1988; Hsieh et al. 1989*b*), and none of them have been mapped near *NCF1* or *NCF2*. However, the gene for human cytochrome P-450 reductase (*POR* locus) has been mapped to the same band (7q11.2) as has *NCF1* (Shephard et al. 1989). The POR gene product is a flavoprotein component of the cytochrome P450–mediated mono-oxygenase of microsomal membranes. It transfers electrons from NADPH to the various cytochrome P450s and thus plays a major role in both detoxification of exogenous compounds and metabolism of endogenous substrates. There is no significant sequence similarity among these two colocalized genes whose products may carry out similar functions.

The *NCF2* gene maps to the same region as does *OTF1*, the gene for the ubiquitous octamer-binding protein Oct-1, on human and mouse chromosome 1 (Hsieh et al. 1990). This region of conserved synteny on human 1q21-q32 and distal mouse chromosome 1 now contains more than 10 homologous loci (Lalley et al. 1989; Seldin and Kruh 1989; Hsieh et al. 1990). Their orientation with respect to the centromere is inverted in the two species. Loci closest to *NCF2* in 1q25 are the gene for laminin B2 (*LAMB2*) and *ABLL*, an Abelson virus–related gene and member of the tyrosine ki-

Table 4

Recombinant Inbred (AK × D) Strain Distribution Pattern of Chromosome 1 Loci in 23 Strains

	AK × D Strain Number																						
	0	0	0	0	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2
Locus	1	2	3	6	7	8	9	0	1	2	3	5	6	8	0	1	2	3	4	5	6	7	8
Ncf-2	A	D	A	D	D	A	D	D	A	D	A	A	D	A	D	A	D	D	A	D	A	A	D
Xmv-21[a]	A	D	A	D	D	A	D	D	A	D	A	A	D	A	D	A	D	D	A	D	A	A	D

[a] Data are from Frankel et al. (1989).

Table 5

Classification of CGD Forms on the Basis of Mutant Gene Locus

Inheritance	Cyt b	Locus	Protein	Location	RFLP	MIM Number	GenBank Accession Number
XLR.....	neg (rare pos)	CYBB	gp91*phox*	Xp21.1	*Nsi*I[a]	306400	X04011
AR.....	pos	NCF1	p47*phox*	7q11.23		233700	M25665
AR.....	pos	NCF2	p67*phox*	1q25	*Hin*dIII[b]	233710	M32011
AR.....	neg	CYBA[c]	p22*phox*	16[d]		233690	J03774

[a] Source: Battat and Francke (1989).

[b] Source: Kenney et al. (1990).

[c] Refers to the α subunit of cytochrome b_{558}. The assignment of this gene to chromosome 16 is mentioned in the MIM entry but has not been formally reported.

[d] Source: Dinauer et al. (1989*a*).

nase family (Kruh et al. 1986; Seldin and Kruh 1989), and possibly α spectrin (*SPTA*). While in human a single locus for NCF2 was found, the mouse genome contains two sites of hybridization to the NCF2 probe. In addition to *Ncf*-2 on chromosome 1, a single, most strongly hybridizing restriction fragment was mapped to chromosome 13. Most likely, this represents a processed pseudogene.

The precise localization of *NCF1* and *NCF2* has implications for prenatal diagnosis of AR-CGD. Even though homozygotes for mutations at these loci may be clinically less affected than patients with classical X-CGD (Clark et al. 1989) and although superoxide production of phagocytes with p47^{phox} deficiency can be greatly improved by γ interferon treatment (Sechler et al. 1988), the identification of affected fetuses may still be desirable in some families. Previously, fetal blood sampling was the only way to make a prenatal diagnosis of CGD. DNA-based studies of chorionic villus material could make use of gene probes that recognize either linked polymorphisms or the mutational change directly. If that is not possible, flanking RFLP markers can be designed on the basis of the map of location of the affected gene loci reported here.

Acknowledgments

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