Brief Definitive Report

Mapping of the *nu* Gene Using Congenic Nude Strains and In Situ Hybridization

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Summary

The chromosomal location of the *nu* gene, which is responsible for hairlessness and athymus, was determined using six DNA markers (interleukin 3 [Il-3], Myhs, Acrb, Evi-2, Mpo, and Hox-2) on mouse chromosome 11. We constructed the high-resolution physical mapping of the six DNA markers on chromosome 11 by in situ hybridization using fluorescence-labeled cosmid probes. The results indicate the order of centromere-(41cM)-Il-3-(3cM)-Myhs-(4cM)-Acrb-(6cM)-Evi-2-(3cM)-Mpo-(5cM)-Hox-2. We have used congenic nude strains and examined which of the six DNA markers were derived from the original nude mouse. We found the Evi-2 locus is linked to the *nu* gene in all the informative, independent congenic nude strains. From these data, we could estimate the location of the *nu* gene, not only genetically but also physically within a region that spans ~17 megabases (9 cM) between the Acrb and Mpo genes.

M utations in the *nu* gene cause the phenotypes of hairlessness and athymus. The embryonic default of thymic development results in the absence of T lymphocytes and severe immune deficiency (1). The nude mutation was originally found in a European closed colony of not completely inbred albino mice (referred to as the original nude mouse in this study), and then transferred into a BALB/c inbred strain by back-crossing several times, giving rise to the present BALB/c-nude mouse. During this step, the nude phenotype was proven to be due to a single locus mutation that is inherited in an autosomal recessive manner (2), and localized genetically to the mouse chromosome 11 by linkage to two mutations, Re and Tr (2, 3). Using the BALB/c-nude mouse, C(D)nu, as the donor strain, several congenic nude strains were established by back-crossing and/or cross-intercrossing with several inbred mouse strains as the inbred partner, through the selection of the nude phenotype as the differential locus (4). In theory, a marker that is linked to but separated from the *nu* gene by X cM on the original nude mouse chromosome will be retained by the probability of $1 - X/100$ at each back-crossing. After as many as 20 back-crosses in the case of the congenic nude strains used in this study, the chance that a marker 14 cM apart from the *nu* gene is still retained is about 5%. Thus, the congenic nude strains should carry only about a 10-cM chromosomal segment containing the *nu* gene that was derived from the original nude mouse.

Although the *nu* gene has been thus mapped genetically, its precise position relative to DNA markers has been unknown. In view of the total absence of information about the *nu* gene product, the "reverse genetical" approach, in which the chromosomal position of a target gene is first assigned by linkage analysis, is one of the most direct and hopeful methods to identify the *nu* gene. In general, the chromosomal assignment of a mutant gene and the search for closely linked DNA markers require painful and time-consuming steps to study RFLPs of hundreds of progeny DNAs using many DNA markers. To ease these burdens, we took advantage of using several congenic nude strains, each of which retains only the small chromosomal segment containing the *nu* gene derived from the original nude mouse and that carries most of the genome derived from the inbred partner strain. We screened the DNA markers that are linked to the *nu* gene by analyzing RFLPs among three independent congenic nude strains. We also used high-resolution fluorescence in situ hybridization (FISH) to determine the physical localization of the DNA markers. This strategy allowed us to reevaluate the previous *nu* gene mapping efficiently and to map the *nu* gene within 9 cM between the Acrb and Mpo genes.

Materials and Methods

FISH. FISHs were carried out with fluorescence-labeled mouse cosmid DNA probes under the condition that suppresses signals from repetitive DNA sequences as previously described (5). Chromosomes were observed by using Laser Sharp MRC-500 (Bio-Rad Laboratories, Richmond, CA). All cosmid clones except for Hox-2 were cloned from an AKR/J DNA cosmid library which was constructed as described (6). Hox-2 cosmid and cDNA clones were given by N. Takahashi, Nagoya University, Nagoya, Japan (7).

Southern Blotting Hybridization. Probes used for Southern blot hybridization are the 360-bp HindIII-XbaI fragment of the mouse IL-3 cDNA clone (8), the 633-bp PstI fragment of the mouse myosin heavy chain (Myhs) cDNA clone (9), the 600-bp BamHI fragment of the bovine acetylcholine receptor β chain (Acrb) cDNA clone (10), the 500-bp PstI fragment of the mouse ecotropic virus integration site 2 (Evi-2) genomic clone (11), the 2.2-kb EcoRI fragment of the mouse myeloperoxidase (Mpo) cDNA clone isolated through crosshybridization with the human Mpo cDNA (12), and the 2.0-kb EcoRI fragment of the mouse homeobox-2 (Hox-2) cDNA clone (7). The probes were purified by agarose gel electrophoresis and labeled with ^{32}P by the random hexamer priming method. High molecular weight DNAs were prepared from livers of each strain as described (13). Restriction digestion, electrophoresis, and Southern blot hybridization were done as described (14). Autoradiograms were taken on an x-ray film (Rx; Fuji Film, Tokyo, Japan) using an intensifying screen, or on an imaging plate for analysis with a Bio-Image Analyzer (BAS2000; Fuji Film, Tokyo, Japan). Restriction enzymes and α-$[^{32}P]$-dCTP were obtained from Takara Shuzo Co. (Kyoto, Japan) and Amersham Japan (Tokyo, Japan), respectively.

Mice. AKR/Ms congenic nude strain (Anu), its inbred partner strain (AKR/Ms), and BALB/c-nude mice (C(D)nu) were maintained by H. Shisa. The congenic strain of BALB/c nude (KITASATO) (CnuK) was established by back-crossing 20 times with BALB/c strain and was maintained by N. Osawa. C57BL/6N congenic nude strain (Bnu) and its inbred partner strain (C57BL/6N) were obtained from M. Saito (Central Institute for Experimental Animals, Kawasaki, Japan). Rb(4,11)12Rma mouse line was obtained from The Jackson Laboratory, Bar Harbor, ME.

Results and Discussion

Since the *nu* gene was genetically assigned around the middle of the long arm of mouse chromosome 11, we chose six DNA markers for the reference of the nude locus. Il-3, Myhs, Acrb, Evi-2, Mpo, and Hox-2, were also assigned around this region. Although these markers have been aligned on chromosome 11 in order (15), the accuracy of the previous loci assignments and genetic linkage maps is limited because they were generated by a simple accumulation of the data based on independent genetic crosses and such data contain fluctuating errors ascribed to a relatively small number of recombinants. An inaccurate distance between the markers may have led to the misalignment of their order. It is well known that genetical distance based on the frequency of the recombination does not always correlate with physical distance on DNA because of hot spots or silent regions of recombination. It is therefore important to know the relative physical locations of DNA markers on the chromosome. For this purpose, in situ hybridization is the most feasible way, but its limit of resolution and identification of mouse chromosomes, all of
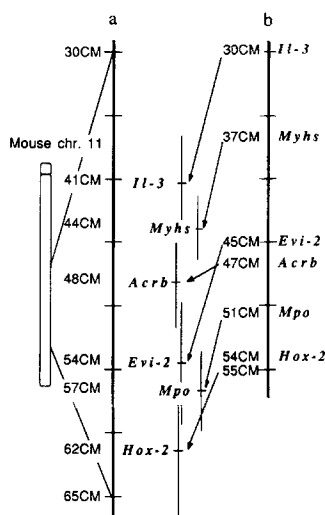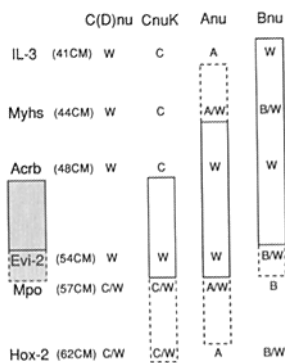

Figure 1. Physical chromosomal map of mouse chromosome 11 by FISH. (*a*) All distances (cM) are calculated from FISH data shown below, and assume the length of mouse chromosome 11 is 78 cM. Distance (%) ± SD of markers indicated from the centromere in proportion to the length of the long arm are as follows: Il-3, 52.0 ± 4.8 (*n* = 28); Myhs, 56.2 ± 3.2 (*n* = 29); Acrb, 61.8 ± 4.7 (*n* = 46); Evi-2, 69.8 ± 6.2 (*n* = 35); Mpo, 72.6 ± 4.0 (*n* = 30); Hox-2, 79.1 ± 6.9 (*n* = 26). (*n*) No. metaphases analyzed. (*b*) The map of mouse chromosome 11 has recently been published (15). (*Arrows*) Relative positions of the same markers in the two mappings.

which are similar in shape and size are two difficulties. The former was overcome by combining the FISH technique and analysis using a laser scanning microscope that could achieve a sub-megabase or sub-centimorgan level of resolution (5). We have avoided the latter problem by the use a mouse line that carries a Robertsonian translocation between the 4th and 11th chromosomes, Rb(4,11)12Rma.

Our map of the six DNA markers constructed by the FISH method coincided roughly with that published previously (15), except for the order of Acrb and Evi-2 (Fig. 1). Physical distances between the markers (tentatively expressed in cM) were calculated assuming that the total length of the mouse chromosome 11 is 78 cM (15) and that the physical distance from the centromere determined by FISH linearly corresponds to the genetic length (Fig. 1). This calculation may not fit the genetic length obtained from recombination frequency, but is probably a better estimate of the physical distance. Consideration of the synteny between human and mouse also sup-
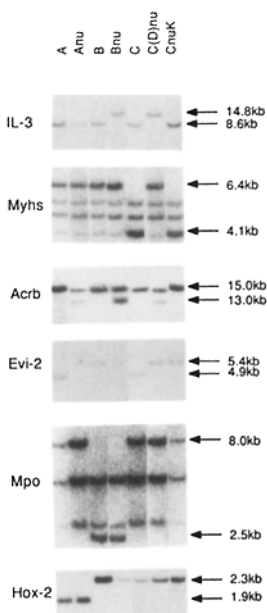
Figure 2. RFLP patterns among inbred partners and congenic nude strains. (←) indicate polymorphic bands. *A*, *B*, and *C* indicate AKR/Ms, C57BL/6N, and BALB/c inbred strains, respectively. Restriction enzymes used for the detection of RFLPs of Il-3, Myhs, Acrb, Evi-2, Mpo, and Hox-2 are EcoRI, AccI, EcoRI, StuI, AccI, and HindIII, respectively.

C(D)nu CnuK Anu Bnu

	C(D)nu	CnuK	Anu	Bnu
IL-3 (41CM)	W	C	A	W
Myhs (44CM)	W	C	A/W	B/W
Acrb (48CM)	W	C	W	W
Evi-2 (54CM)	W	W	W	B/W
Mpo (57CM)	C/W	C/W	A/W	B
Hox-2 (62CM)	C/W	C/W	A	B/W

Figure 3. Chromosomal segment retained by congenic nude strains. Abbreviations of mouse strains are as indicated in Fig. 2. W, A, B and C indicate DNA derived from the original nude mouse, AKR/Ms, C57BL/6N, and BALB/c, respectively. (Solid boxes) Chromosomal segments derived from the original nude mouse. (Broken boxes) Chromosomal segments whose origins were not determined. (Shaded box) Region commonly retained by all congenic nude strains.

ports the order because ACHRB is located on the short arm of the human chromosome 17 (reference 15 and our unpublished observations), just as MYHC (15) and EVI2 are located on the long arm of the human chromosome 17 (16), as is MPO (15).

Three congenic strains, BALB/c nude (KITASATO) (CnuK), AKR/Ms nude (Anu), and C57BL/6N nude (Bnu) were chosen for our analyses. We have tested DNAs of Anu, Bnu, and CnuK, in addition to their inbred partners, i.e., AKR/Ms, C57BL/6N, and BALB/c, and the nude donor strain, C(D)nu, using the DNA probes. We found informative RFLPs between C(D)nu and at least one inbred partner strain for all six DNA markers (Fig. 2). Since the C(D)nu strain, which was back-crossed only several times, probably carries a wide region of chromosome 11 derived from the original nude mouse, we have assumed that a polymorphic difference between C(D)nu and BALB/c mice is due to DNA derived from the original nude mouse.

The IL-3 probe detected the 14.8-kb EcoRI band in the DNAs of C(D)nu and Bnu mice, but the 8.6-kb EcoRI band in DNAs of the other mice. Since there is a polymorphic difference between BALB/c and C(D)nu mice, the 14.8-kb EcoRI band in C(D)nu and Bnu mice must have been derived from the original nude mouse DNA. The Il-3 loci of CnuK and Anu mice are likely to be derived from their inbred partners. The Myhs probe detected the 4.1-kb AccI band in DNAs of BALB/c and CnuK, and the 6.4-kb AccI band in the DNAs of all the other mice including C(D)nu. The Myhs genes in C(D)nu and CnuK mice are therefore likely to be derived from the original nude and BALB/c mice, respectively. However, the origins of the Myhs loci in Anu and Bnu mice could not be definitely determined because we could not distinguish the Myhs locus among C(D)nu and AKR/Ms, C57BL/6N mice. The Acrb probe distinguished Anu, Bnu, and C(D)nu DNAs from the rest by detecting an additional 13.0-kb EcoRI band. The results indicate that C(D)nu, Anu, and Bnu DNAs have the Acrb gene derived from the original nude mouse whereas the Acrb gene of CnuK DNA already has been assimilated to the inbred partner BALB/c type. The Evi-2 probe showed that DNAs of AKR/Ms and BALB/c mice were polymorphically different from those of the rest, including C(D)nu. The result indicates that the Evi-2 loci of C(D)nu, CnuK, and Anu are derived from the original nude mouse, but the origin of the Evi-2 locus in Bnu mice is not definitive. The Mpo probe identified a 2.5-kb poly-

morphic StuI band only in C57BL/6N and Bnu DNA. The result indicates that Bnu DNA has the C57BL/6N-type Mpo gene but the Mpo probe did not provide the other congenic strains with the useful information. The Hox-2 probe detected a 1.9-kb polymorphic HindIII band only in AKR/Ms and Anu DNA, indicating that Anu DNA has the AKR/Ms-type Hox-2 gene. The Hox-2 probe was not informative for the origin of the Hox-2 gene in CnuK and Bnu mice. As the Mpo and Hox-2 loci of C(D)nu were indistinguishable from those of BALB/c, we could not tell whether these loci of C(D)nu mice were derived from the original nude mice or not. These results are summarized using the gene order determined above (Fig. 3).

We found that RFLP patterns of four of six markers were different between BALB/c and C(D)nu mice. RFLP patterns of four markers of C(D)nu are still the original nude mouse type, but only a part of them are retained by the congenic strains as expected. For example, CnuK mouse retains a smaller region than C(D)nu mouse as RFLP patterns of the Il-3, Myhs, and Acrb genes were shown to be identical to those of the inbred partner strain, BALB/c. Each congenic mouse strain retained a reasonable portion of chromosome 11 derived from the original nude mouse. The retained region by each congenic nude strain overlapped with each other surrounding the Evi-2 locus (Fig. 3). The nu gene should lie in the region that is transferred from the original nude mouse to all the congenic nude strains. We therefore conclude that the nu gene lies within the 17-Mb (9-cM) region between the Acrb and Mpo genes (excluding themselves), including the Evi-2 locus. We confirmed this conclusion by analyzing DNAs of offsprings by the cross between C(D)nu homozygote and C(D)nu-BALB/c heterozygote. Only 3 of 100 mice with the nude phenotype showed the heterozygosity at the Evi-2 locus, indicating that the distance from the Evi-2 locus to the nu locus was 3 cM (data not shown).

Another possible approach in identifying unknown genes responsible for genetic disorders is to use chromosomal synteny. The murine gene mapping may be easily accomplished by confirming human gene mapping, which usually has more abundant markers. In this case, however, this strategy is not such a straightforward approach. The EVI2 and ACRB genes are located on the long and short arms, respectively, of the human chromosome 17 (15, 16, and our unpublished observations), indicating that the mapping of human DNA markers surrounding these two loci does not necessarily correlate with that of murine homologues. We could not find any obvious candidate genes of the nude mutation that map to the EVI2 and ACRB regions of human chromosome 17.

We have determined the chromosomal location of the nu gene relative to DNA markers, not only genetically, but also physically by the combination of FISH and RFLP analyses of congenic nude strains. In reverse genetics, a mutant locus is usually mapped by an intersubspecies phenotypic backcrosses, which requires a lot of time and efforts, but the use of congenic strains of a mutant provides a faster and more efficient way of mapping. We also showed that the combination of physical and genetic evaluation of chromosomal mapping is very important to avoid misalignment of the gene order.

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