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Nackt (*nkt*), a new hair loss mutation of the mouse with associated CD4 deficiency

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Abstract A spontaneous recessive mutation named nackt (symbol: *nkt*) affecting hair growth and T-cell development was discovered in a moderately inbred stock of mice. Skin lesions were characterized by sparse rough coat, bare patches around the eyes and neck, and a scratching behavior throughout life. Fluorescence-activated cell sorter analysis indicated a deficiency in the $CD4^+$ 8^- T-cell subset in the thymus and a marked decrease in $CD4^+$ T cells in peripheral lymphoid organs. Linkage analysis using a set of molecular markers and an F_2 intersubspecific cross indicated that the mutation maps to the central region of mouse chromosome 13, in a region homologous to human chromosome 5q22-q35.

Key words $CD4^+$ deficiency · Hair-loss mutation · Laboratory mouse

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Introduction

Mutations affecting the integument (skin and hair texture or coat color) are abundant in the laboratory mouse (Doolittle et al. 1996). In general these mutations, because of the nature of their phenotype, are relatively easy to detect and do not severely impair the viability of affected animals. The integument is a rather complex tissue from an embryological point of view and many of these mutations have pleiotropic effects and are accordingly potentially useful tools for elucidating the underlying developmental mechanisms. In some instances the skin phenotype, although most obvious, appears relatively secondary when all the pleiotropic effects are inventoried. Several such mutations have proven to be homologous to specific human diseases and represent useful animal models (Sundberg 1994).

Here we report a novel mouse mutation designated nackt (symbol *nkt*). Homozygous mice exhibit a relatively severe fur deficiency associated with congenital maturational arrest of $CD4^+$ 8^- formation but not $CD4^-8^+$ from $CD4^+8^+$ cells. To our knowledge, this is the first report of a spontaneous mutation in the mouse associated with CD4 deficiency. Although nothing is known concerning the molecular basis of this mutation, it represents a potentially interesting tool for investigating the mechanisms of selection operating in the thymus.

Materials and methods

Mutant allele and mouse strains

The *nkt* mutant allele arose in 1981, in the laboratory animal facilities of the Martin-Luther-Universität in Halle-Wittenberg (Germany) in a stock derived from irradiated founder N° 372 of the late P. Hertwig. A pair of mutant mice were introduced into the animal facility of the Institut Pasteur in 1991, where they were crossed to 129/Sv//Pas inbred breeders for a few generations, then intercrossed (this partially congenic strain will be designated NKT/Pas hereafter). Mice segregating for *nkt* were finally transferred to the animal facilities of the Instituto de Investigaciones Hematológicas, Buenos Aires, where the mutant allele was backcrossed onto three different backgrounds (BALB/c, C57BL/6, and DBA/2) by the performing, in each case, of four successive rounds of cross-intercross (N4).

For linkage analysis, an intersubspecific intercross (F_2) was set up using the MAI/Pas inbred strain of *Mus musculus musculus* origin (Bonhomme and Guénet 1996). Such an intersubspecific intercross increases the amount of polymorphism segregating in the progeny (Guénet et al. 1988) and helps greatly in the detection of linkage.

For immunological studies, specific pathogen-free (SPF) animals were obtained by cesarean derivation of BALB/c-*nkt* (N4) and C57BL/6-*nkt* (N4) mice at the laboratory animal facilities of the Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina. These mice were maintained under SPF conditions in micro-isolators and were handled using sterile techniques in laminar flow hoods.

Flow cytometric analysis

Thymocytes were prepared from mice (1-day to 9-months old) and stained with both fluorescein isothiocyanate-conjugated mouse-specific CD4 (RM4-5 clone, Pharmingen, San Diego, CA.) and PE-conjugated mouse-specific CD8 monoclonal antibodies (mAbs) (53-6.7 clone, Pharmingen) for two-color staining. Lymph node cells were prepared from axillar and submandibular lymph nodes (LN) and stained by the two-color procedure described above. Mononuclear cells from peripheral blood and spleen were isolated by centrifugation on Ficoll-Triyosom gradients (density = 1.09 g/ml). Analysis were performed with a FACScan (Beaton-Dickinson, Franklin Lakes, NJ) and the Cell Quest program (Becton-Dickinson). Dead cells were gated out by forward and side light scatters.

Histological analysis

Skin samples were obtained from the dorsal and ventral parts of the body as well as from the head, ear, eyelid, foot pad, tail, and tongue of normal (+/*nkt*?) and mutant (*nkt*/*nkt*) littermates on a BALB/c and C57BL/6 background at days 21, 30, 90, and 180. After fixation in Fekete and standard paraffin embedding, 3–5 μ thick sections of harvested skin were stained with hematoxylin-eosin (H.E.) and Giemsa. Single hairs were also observed directly under optical microscope.

Linkage analysis

DNA samples were prepared from the spleen of 40 F_2 mice homozygous for the *nkt* mutant allele using the classical phenol/chloroform method. Fifty-two microsatellite markers, evenly distributed over the whole genetic map, were selected (Aitman et al. 1991; Dietrich et al. 1996; Montagutelli et al. 1991). For accurate localization, the four closest flanking molecular markers to the *nkt* locus were mapped using the European Collaborative Interspecific Backross (or EUCIB) DNA resource prepared from an interspecific backcross progeny of the type (C57BL/6 × *Mus spretus*)F_1 × C57BL/6 (Breen et al. 1994). A subset of 220 DNA samples from the EUCIB panel, corresponding to informative recombinant haplotypes between molecular markers *D13Mit138* and *D13Mit28* (spanning 28 cM of the mouse chromosome 13 consensus genetic map by Justice and Stephenson 1997), were used for a better localization of the *D13Mit247* and *D13Mit25* loci. Microsatellites were amplified from 100 ng of template DNA in a final reaction volume of 25 μl containing 10 mM Tris-HCl, pH 8.0, 1–2 mM $MgCl_2$, 0.2 mM deoxynucleotides, 0.1% Tween 20, 125 ng of each primer, and 0.6 to 1.25 Units of Ampli *Taq*-Polymerase (Amersham, Uppsala, Sweden). The primers used in this experiment were all purchased from Research Genetics (Cleveland, OH). The samples were covered with mineral oil (Sigma™, St Louis, Miss.) and amplified with a Techne (Hampton, NH) PHC-3 apparatus. After initial denaturation (94 °C for 4 min), 35 cycles of amplification were performed (denaturation 94 °C for 40 s; annealing 52–55 °C for 40 s) and a final elongation step of 5 min at 72 °C was performed. Amplification products were scored on 4% NuSieve agarose gels with classical ethidium bromide staining. Statistical analysis of haplotype segregations were performed manually. The linear ordering of molecular markers and the computation of the distances between these markers in the EUCIB/DNA resource were performed using the Gene Link software (Montagutelli 1990).

Results

Alopecia and dermatitis

Mutant mice have relatively normal fur up to the age of 12 days. From this age, the coat begins to become sparse and rough and the mice start scratching, which suggests that they are suffering from chronic pruritus. Diffuse alopecia with completely bald patches around the neck and eyes is a constant feature during the entire life span of the animals, as is the pruritus (Fig. 1A). When maintained in non-SPF facilities, *nkt*/*nkt* mice develop ulcerative skin lesions which, in adults, are always associated with enlargement of the regional lymph nodes (preferentially in the head, neck, and hind limbs). This dermatopathic lymphadenitis is associated with cases of generalized exfoliative dermatitis.

Histologically, the epidermis appears hyperplastic with acanthosis, hypergranulosis, epidermal, and follicular orthokeratosis (Fig. 1B, C, D). The dermis shows infiltration of inflammatory cells, such as lymphocytes, mast cells and polymorphonuclear cells. There is also hypertrophy of the sebaceous gland which is, presumably, a consequence of a discrete dermatitis. Skin lesions are consistently less severe in the ventral region than in other parts of the body. Homozygous *nkt*/*nkt* mice frequently develop opacification of the cornea with a somewhat heterogeneous phenotype.

Affected mice of both sexes have normal hematological and biochemical parameters and no clinical features other than those mentioned above have been observed. The *nkt*/*nkt* mice of both sexes are fully fertile when bred under SPF conditions.

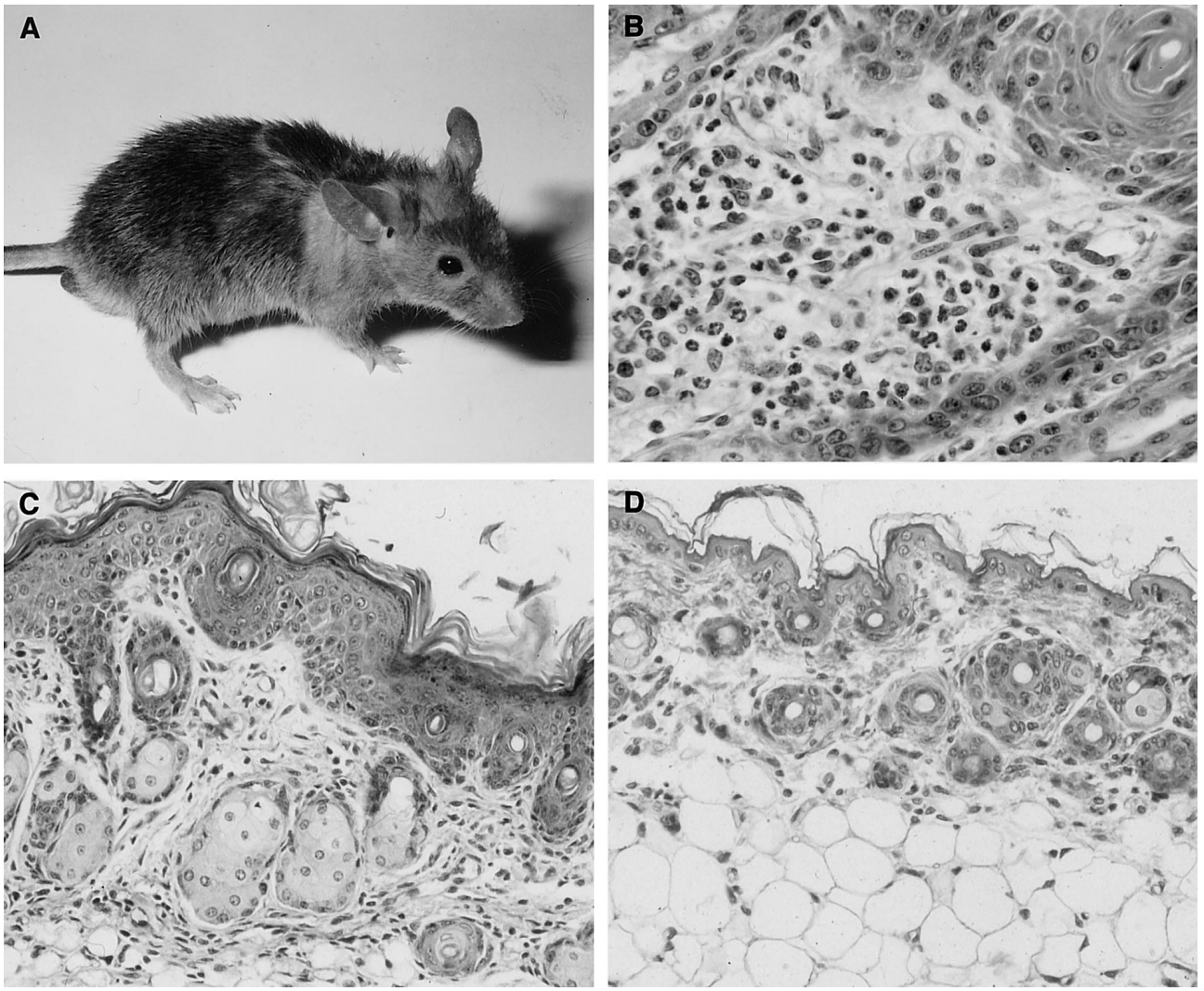


Fig. 1 **A** Alopecia in an adult NKT/Pas – *nkt/nkt* mouse. When aged 3 to 4 weeks, homozygous mice are completely hairless. **B, C, D**: Histological findings at 60 days of age. **B** Infiltration of the dermis with lymphocytes, plasmocytes and polymorphonuclear cells in a BALB/c – *nkt/nkt* mouse. **C** Hyperplastic lesions of the epidermis in the same animal (acanthosis, hypergranulosis, epidermal and follicular orthokeratosis). Notice also sebaceous hypertrophy. **D** BALB/c – +/*nkt*? mouse: normal skin. Original magnification × 400 (**B**), × 200 (**C**, **D**)

Deficiency in CD4$^+$ T cells

When homozygous *nkt*/*nkt* mice were bred in a conventional environment, with enzootic mouse hepatitis virus (MHV) infection, we observed that growth was much retarded and that the affected animals developed a wasting syndrome by the age of three weeks. Neonatal mortality rate in mutant mice nursed by an immunocompetent +/*nkt* dam was high (50%) at weaning, in comparison with the mean value for the animal facility (around 1%). In addition, *nkt*/*nkt* offspring nursed by *nkt*/*nkt* mothers exhibited a very high neonatal mortality rate since, by day 10 post partum, the majority of the pups were dead. Histopathologic studies revealed hepatitis and thymic atrophy with an inverted cortical image. MHV infection was confirmed by immunofluorescence. These observations were suggestive of an immunodeficiency of the *nkt*/*nkt* mutant mice against natural MHV infection in which it has been reported that virus-specific CD4$^+$ CTL play a pivotal role (Heemskerk et al. 1995).

Taking these observations into account and using SPF, MHV negative *nkt*/*nkt* mice, we examined the distribution of T-cell subsets in different lymphoid tissues. Table 1 and Fig. 2 show the results obtained in BALB/c-*nkt*/*nkt* mice (N4) and their normal littermates. The most striking finding was a marked decrease in the proportion of CD4$^+$ T cells in three of the four lymphoid tissues examined. Similar results were obtained in partially congenic strains C57BL/6-*nkt* (N4) and DBA/2-*nkt* (N3). This decrease in the CD4$^+$ subset is absolute and not relative because the number of splenocytes, thymocytes, and blood cells was found to be very similar in *nkt*/*nkt* and +/*nkt*? mice. In contrast, the number of cells in the lymph nodes of mutant animals is about fourfold higher than that of normal mice, which makes

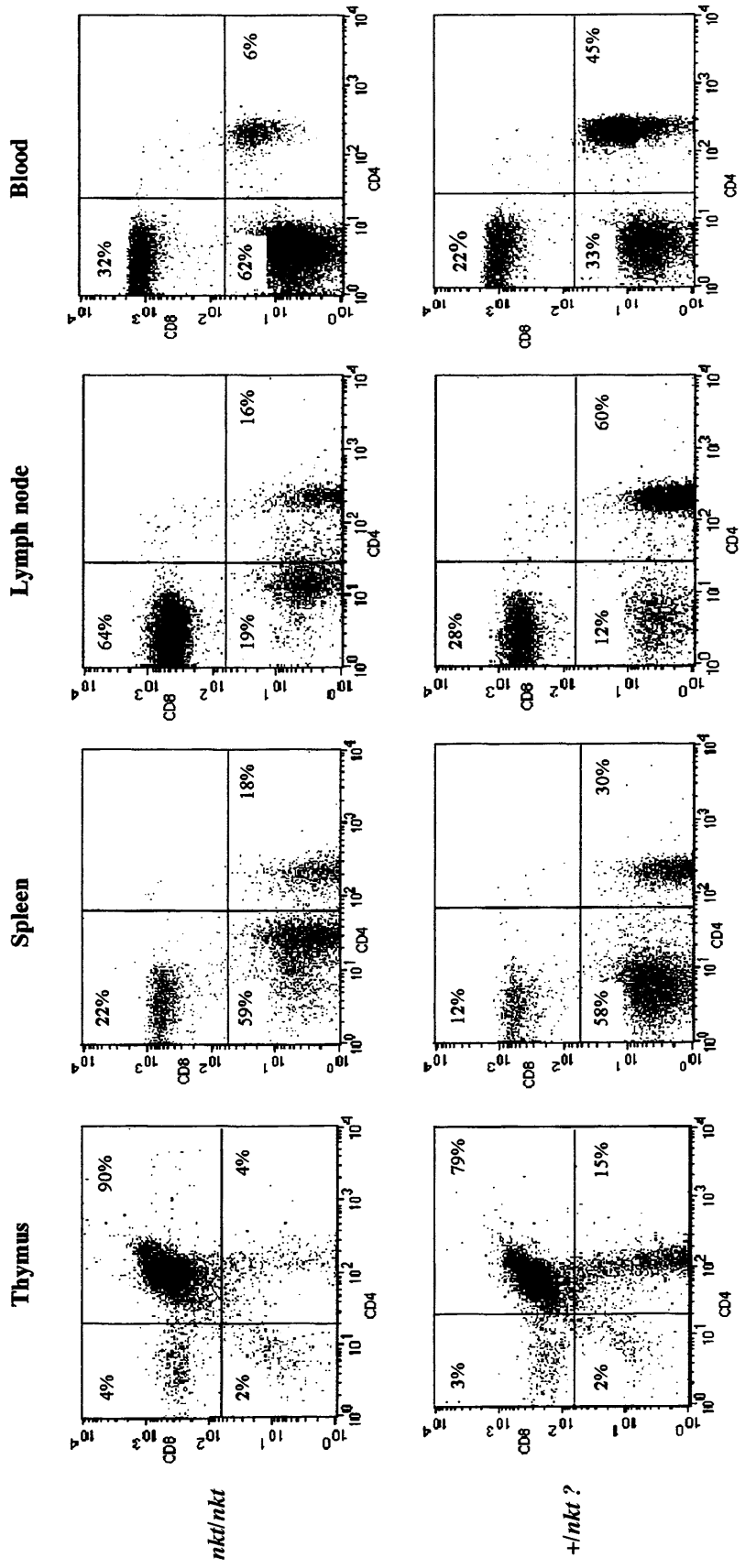


Fig. 2 FACS analyses of CD4/CD8 antigens on thymocytes, spleen cells, lymph nodes cells, and PBL in specific pathogen-free (SPF) BALB/c-*nkt*/*nkt* mice

Table 1

	Thymus				Lymph nodes		Spleen		Blood	
	CD4⁻8⁻	*CD4⁺8⁻*	*CD4⁻8⁺*	*CD4⁺8⁺*	*CD4⁺*	*CD8⁺*	*CD4⁺*	*CD8⁺*	*CD4⁺*	*CD8⁺*
nkt/nkt	6.4±0.7%	**2.6±0.3%**	7.3±0.6%	82.8±5.5%	**8.8±0.8%**	**51.9±2.3%**	20.7±1.5%	**20.2±2.7%**	**3.7±0.8%**	24.0±5.2%
+/nkt?	3.5±0.3%	10.5±0.5%	4.9±0.6%	81.0±4.7%	44.3±2.3%	23.7±1.2%	29.0±1.0%	9.6±1.2%	36.3±4.6%	18.0±4.3%

the shortage in CD4$^+$ lymphocytes less obvious. It is noteworthy that the CD4$^+$ deficiency was observed in neonatal animals and persisted for at least nine months.

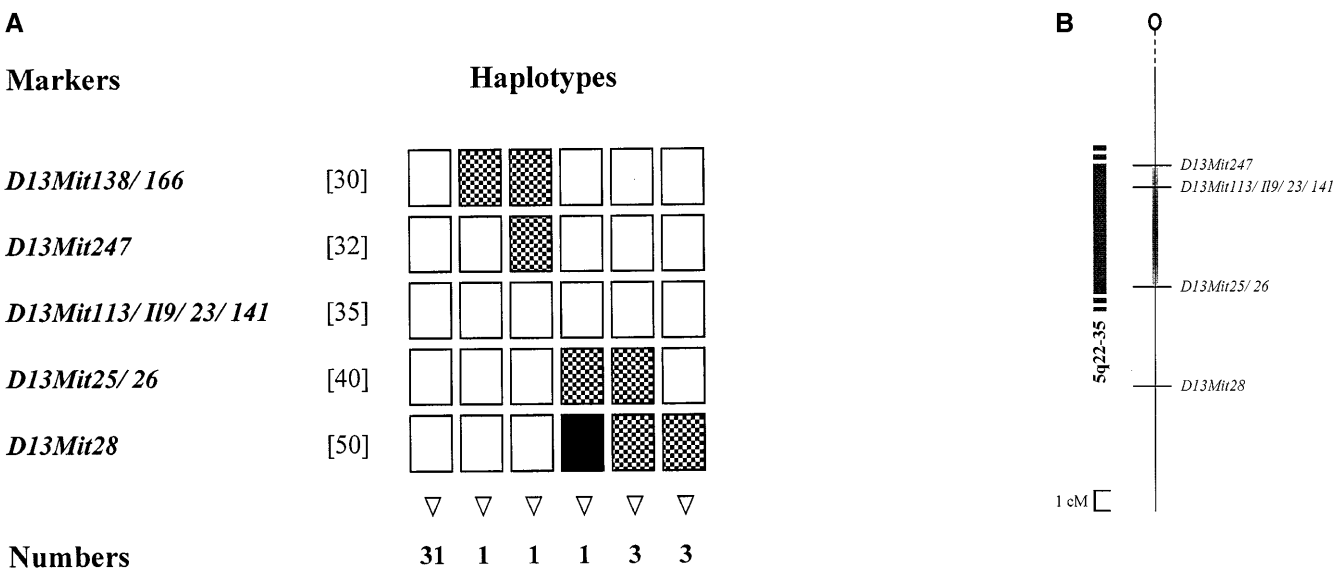
Nackt is an autosomal recessive mutation with complete penetrance

All F_1 offspring (160) born to C57BL/6, DBA/2, and BALB/c inbred mothers when crossed with *nkt/nkt* males during the derivation of congenic strains (see above) were found to have a normal phenotype. In the merged population of the three F_2, 23.5% of the offspring (32 of 136 mice) exhibited fur deficiency typical of the [nackt] phenotype, a proportion which is not statistically different from the theoretical proportion (25%) expected for a single recessive mendelian character. No linkage with sex was detected. Crossing several *+/nkt* F_1 females back to *nkt/nkt* males yielded 167 progeny 77 of which (46.1%) were found to be affected, a proportion which, here again, closely matches the theoretical 50% proportion expected in a backcross.

The nkt locus maps to mouse chromosome 13

A set of 52 microsatellite markers, polymorphic among the parental strains NKT/Pas and MAI/Pas, were used for systematic screening of the haplotype constitution in a sample of 16 *nkt/nkt* progeny of the intersubspecific (F_2) cross. This systematic genomic scan indicated independent segregation with all chromosomes except chromosome 13 (Chr 13) where a clear segregation distortion was observed for three markers: *D13Mit80*, *D13Mit38*, and *D13Mit47*. When it became obvious that the *nkt* locus was linked to Chr 13, additional microsatellite markers encompassing the critical region were used to screen the 40 *nkt/nkt* offspring of the interspecific cross. These microsatellites were: *D13Mit13*, *D13Mit23*, *D13Mit25*, *D13Mit26*, *D13Mit28*, *D13Mit32*, *D13Mit71*, *D13Mit113*, *D13Mit138*, *D13Mit166*, and *D13Mit247*. A detailed analysis of the haplotypes associated with the most informative markers is shown in Fig. 3A. Among the 40 offspring with a *nkt/nkt* geno-

Fig. 3 **A** Distribution of chromosome 13 haplotypes in the 40 offspring (80 meiosis) of the (NKT/Pas × MAI/Pas) intersubspecific F_2 progeny showing the segregation of markers flanking the *nkt* locus. *White boxes* indicate homozygosity for the allele contributed by the NKT/Pas strain, *black boxes* indicate homozygosity for the MAI/Pas allele, and *hatched boxes* represent heterozygosity (NKT/Pas and MAI/Pas alleles both present). Numbers *between* brackets [30 to 50] indicate distance to the centromere of Chr 13 in centi-Morgans. Numbers below each vertical column indicate the number of F_2 animals showing that haplotype. **B** Location of the *nkt* mutation on mouse Chr 13 (partial). The *shaded box* indicates the region where the *nkt* mutation is likely to map at the 5% risk level. This assignment was made from the data of Fig. 3A. The *gray bar* on the *left* indicates similarity to human chromosome 5 (5q23-35 region)



type, representing a total of 80 meiosis, no recombinants were found with markers *D13Mit23*, *D13Mit113*, and *D13Mit141* as well as with the microsatellite marker *D13Mit13* located at the *Il9* locus (interleukin-9 encoding gene). Haplotype analysis established the following order of loci:

D13Mit138/166 – D13Mit247 – D13Mit23/113/Il9/nkt – D13Mit25/26. This gene order (Fig. 3B), established on a relatively small sized sample, is consistent with the consensus mouse Chr 13 map (Justice and Stephenson 1997) and would indicate that the *nkt* locus maps 30 to 40 cM distal from the centromere.

Discussion

Several genes have been previously mapped to the segment of mouse Chr 13 where the *nkt* locus was assigned [for example: laminin B1 subunit 2 (*Lamb1-2*), calcium modulating ligand (*Caml*), histamine receptor H2 (*Hrh2*), cytotoxic T-lymphocyte-associated protein 2 alpha (*Ctla2a*), Interleukin 9 (*Il9*) and transforming growth factor beta-induced (*Tgfbi*)]. None of these genes, based on their function or expression pattern, appear to be a candidate for the *nkt* mutation. Considering the homology of the chromosomal segment in which the mutation *nkt* was found to map in the mouse, one can predict that the human homologue of the *nkt* mutant allele, should it exist, would map to the q22-q35 region of human chromosome 5 (Fig. 3B). Such a phenotype has however yet to be reported in human.

Several of the mouse mutations with anomalies of the integument associated with more or less severe immunological defects have been reported previously. This is the case for the two mutant alleles at the Winged helix nude locus: Whn^{nu} and Whn^{str}, (formerly nude *nu* and streaker nu^{str}) on mouse Chr 11. Affected mice are hairless from birth and suffer from almost complete thymic agenesis (Flanagan 1966; Shultz et al. 1978). Mice homozygous for the balding allele (*bal*, Chr 18) likewise exhibit both hair growth abnormalities and an increase in the number of mast cells in skin with associated elevated IgE levels (Davisson et al. 1994). Mice homozygous for motheaten mutant alleles (*me*, me^{v}, etc., Chr 6) not only develop severe neutrophilic skin lesions but also show immunodeficiency accompanied by auto-immune disease (Shultz 1988). Finally, hairless mice (*hr* and hr^{rh} alleles, Chr 14) which lose their coat after the first molt, at roughly 14 days of age, have a defective cellular immune response throughout their lives (Reske-Kunz et al. 1979).

In the case of nude mice, the pleiotropic effect is explained by the disruption of the *Whn* gene which is expressed specifically in both skin and thymus. The differentiation of primitive epithelial precursor cells in the thymic primordium into subcapsular, cortical, and medullary epithelial cells of the mature thymus requires the activity of the nude gene product WHN, a transcription factor that is also required for proper keratinization of the hair shaft (Schorpp et al. 1997).

The nackt (*nkt*) mutation represents another mutation with pleiotropic effects on the coat, the eyes, and the immune system. It is distinct from the above-mentioned mutations both because it maps to a different chromosome and because it exhibits a rather different phenotype. The identification of the molecular basis of this mutation requires further study, it is nevertheless particularly interesting to note, as exemplified in the case reported, how one single gene can have dramatic effects on the $CD4^+$ generation pathway. It is clear that the nackt (*nkt*) mutation is likely to become a useful tool for studies not only on the selection mechanisms operating in the thymus but also as an animal model for dermatitis and pruritus.

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