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[1], t t [1], [1], . // [1], c ff [1], t
c [1], / [1], c [2], c ff . t [1] and . [1]

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Two separate lines of investigation have pointed to an important role(s) for Ian proteins in the development of T lymphocytes. First, a differential display study revealed that the expression of mIan1 is strongly induced at or after T cell-positive selection in the thymus; a wave of expression was also detected after the earlier b-selection stage (1). Second, positional cloning of lymphopenia (lyp), which is a major susceptibility locus for type I diabetes mellitus (iddm1) in the diabetes-prone BioBreeding (BB-DP) rat model of this disease (14, 15), has identi ed a frameshift (probably null) mutation in the rIan5 gene as the basis of this trait. In its homozygous state, the lyp mutation is associated with severe peripheral T cell lymphopenia (16, 17) and it has been demonstrated that both peripheral T lymphocytes and mature $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes from lyp/lyp animals are much more susceptible to spontaneous apoptosis than their wild-type (w.t.) counterparts (18, 19).

Given these two independent indications of the importance of Ian genes in T cell development, one in mouse and the other in the rat, we undertook a study of gene expression of the entire Ian gene cluster in lymphomyeloid cells in order to assess expression levels of all Ian family members. We have commenced the study of Ian function by examining the susceptibility to c-irradiation-induced apoptosis of thymocytes and B cells isolated from lymphopenic rats which are homozygous for an Ian5 null mutation. We have developed polyclonal antisera against Ian1 and Ian9, and have used these reagents rst to prove that the Ian9 gene, which is predicted to have an unusual triplicated structure comprising domains encoded by Ian11, Ian10 and Ian9, does indeed produce a corresponding protein product. Second, we determined levels of Ian1 and Ian9 proteins in various lymphoid populations.

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Animals

Rats of the congenic strains PVG-$RT1^uRT7^b$, PVG-$RT1^u$lyp/lyp (18) and PVG (the latter used exclusively as a source of macrophages) and C57BL/6 mice were maintained in speci c pathogen-free conditions in The Babraham Institute Small Animal Barrier Unit. Animals (males and females) were used between 8 and 12 weeks of age. In unpublished analyses we have con rmed that the PVG- $RT1^u$lyp/lyp strain, which derives its mutant lyp gene from the Edinburgh subline of BB-DP rats (20), carries the frameshift mutation in the Ian5 gene described by Hornum et al. (11) and MacMurray et al. (2).

Sample preparation

Various rat primary cell sub-populations were isolated using combinations of mAbs with magnetic bead separations (D]TJ 0 -1.6u1thy cell sub-0(L347 Tcway.428 P6e740(D]y)-356(5ra-352CS

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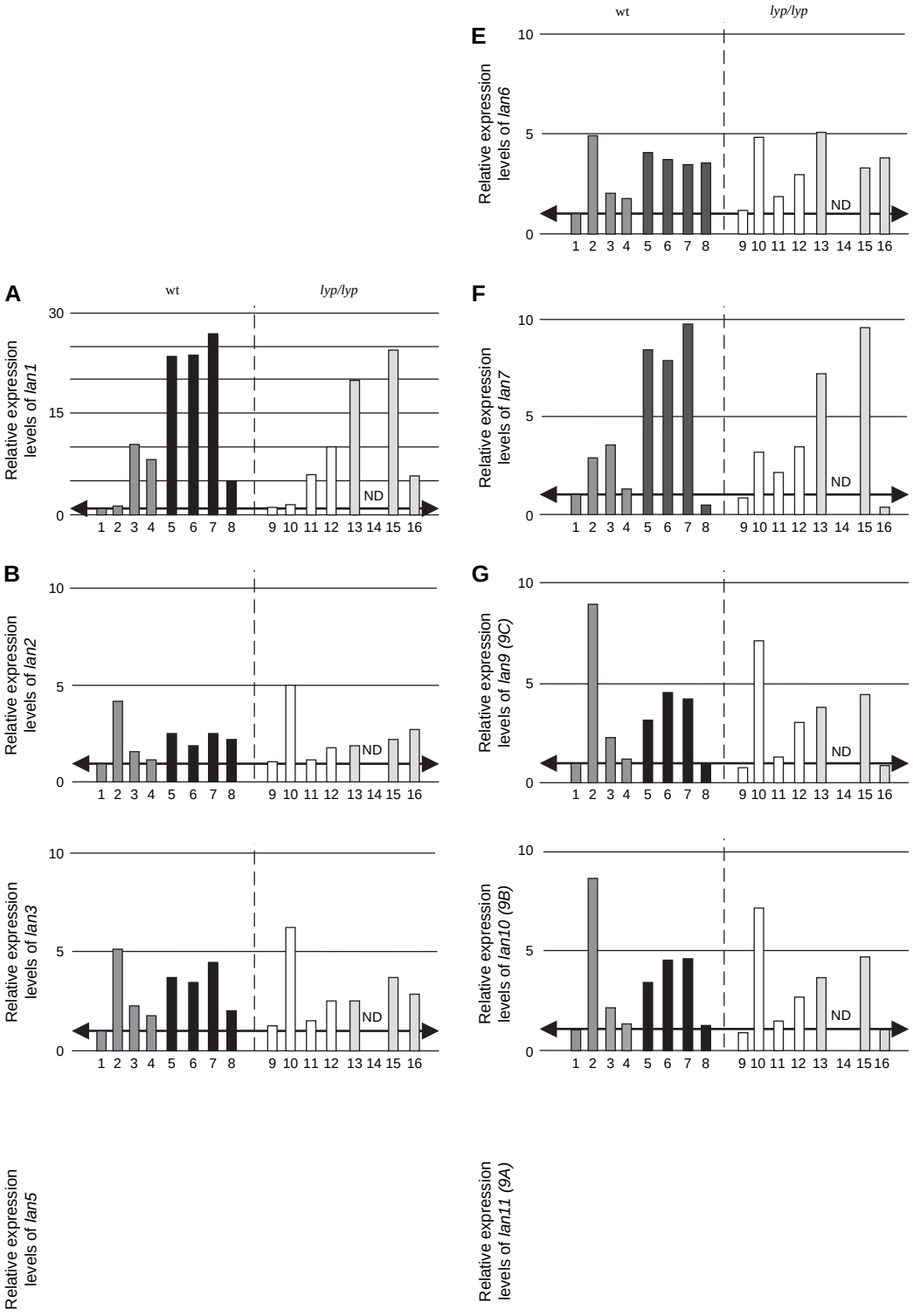
Real-time PCR set up

The two HKGs (6PFKc and cirhin) were selected for their stable expression in the cell populations isolated and studied (Supplementary Data, Figure S1 and Table S1, available at International Immunology Online): the M value, which is a measure of this stability (41), was 0.56 for rat, 0.64 for


A
wt
lyp/lyp
Relative expression levels of lan1
30
15
0
ND
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
Relative expression levels of lan2
10
5
0
ND
C
Relative expression levels of lan3
D
Relative expression levels of lan5
E
Relative expression levels of lan6
F
Relative expression levels of lan7
G
Relative expression levels of lan9 (9C)
Relative expression levels of lan10 (9B)
Relative expression levels of lan11 (9A)

It should be noted that where any expression was seen in cell lines this was generally of the order of expression seen in

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N-terminal third of the Ian9 triple (formerly Ian11); Ian9B, corresponding to the central third of the Ian9 triple (formerly Ian10), and Ian9C, corresponding to the C-terminal third of the Ian9 triple (formerly Ian9). Using trace data from the National Center for Biotechnology Information, we have also been able to assemble a dog Ian9 sequence that lacks probably only a few residues at its amino-terminus. Predicted Ian9 amino acid sequences for human, rat and dog are aligned in Fig. 5(A) where they are compared with an Ian consensus. This alignment compares the three internal 'repeats' within Ian9 (Ian9A, Ian9B and Ian9C) and highlights the conserved elements of the GTPase motif (3, 51). A schematic represen-

It has yet to be determined whether any of the three potential GTP-binding regions of Ian9 is active. Proteins containing multiple sets of guanosine nucleotide-binding domains are highly unusual but a precedent does exist in EngA (and its orthologues), present in all bacterial genomes and Arabidopsis. EngA contains two tandem GTP/GDP-binding domains. The crystal structure of the Thermotaga maritima EngA family member TM-Der has been solved (55). This study is particularly interesting in the context of the Ian9 'triple' because it presents evidence that the two guanosine nucleotide-binding sites of TM-Der are not equivalent. Thus, while both sites have GTPase activity, the more C-terminal site, GD2, has an exceptionally slow intrinsic rate of release of the product GDP. This suggests that the two GTPase domains make different contributions to the regulation of the protein, presumably through different interactions with extrinsic regulators. We are obviously far from knowing the function of Ian9 but the conserved sequence differences that we have pointed out between the three parts of the molecule suggest that similar subtle biochemical mechanisms may be at work as in TM-Der. We noted that in LN T cells, levels of Ian9 protein are reduced when the cells originate from an Ian5-de cient (lyp/lyp) animal. It is noteworthy that the decreases in protein levels of Ian1 and Ian9 observed in lyp/lyp as compared with w.t. LN T cells are discordant with the results obtained on mRNA levels by real-time PCR. This raises the possibility of post-transcriptional regulation of Ian1 and Ian9 expression: previously this has been suggested for hIan1 (3).

On a more general note, it will be important to nd out whether all the Ian family of polypeptides are engaged in the same cellular activity (e.g. apoptosis) or have diverse roles. The eld of known GTPases offers a broad selection of possible functions ranging from the intracellular signaling 'switch', for which ras is a paradigm (51), to involvement in cytokinesis and vesicle traf cking, of which the dynamins and the septins (both carrying coiled-coil domains) are good examples (56, 57).

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Our attention has been drawn to the publication by Kr ücken et al. 2004. Gene 341:291., who showed bioinformatic and northern blot evidence for the existance of the Ian9 'triple'.

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7-AAD	7-amino-actinomycin D
BB-DP	diabetes-prone BioBreeding
DN	double negative
DP	double positive
ER	endoplasmic reticulum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione-S-transferase
h	human
HKG	housekeeping gene
Ian	immune-associated nucleotide
ISH	in situ hybridization
LN	lymph node
lyp	lymphopenia
m	mouse
ORF	open reading frame
6PFKc	6-phosphofructokinase C
r	rat
SIRP	signal regulatory program
SP	single positive
w.t.	wild type

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1 Poirier, G. M. C., Anderson, G., Huvar, A. et al. 1999. Immune-associated nucleotide-1 (IAN-1) is a thymic selection marker and de nes a novel gene family conserved in plants. J. Immunol. 163:4960.

2 MacMurray, A. J., Moralejo, D. H., Kwitek, A. E. et al. 2002. Lymphopenia in the BB rat model of Type 1 diabetes is due to a mutation in a novel immune-associated nucleotide (Ian)-related gene. Genome Res. 12:1029.

3 Cambot, M., Aresta, S., Kahn-Perles, B., de Gunzburg, J. and Romeo, P. 2002. Human immune associated nucleotide 1: a member of a new guanosine triphosphatase family expressed in resting T and B cells. Blood 99:3293.

4 Dahéron, L., Zenz, T., Siracusa, L. D., Brenner, C. and Calabretta, B. 2001. Molecular cloning of Ian4: a BCR/ABL-induced gene that encodes an outer membrane mitochondrial protein with GTP-binding activity.

15 Jacob, H. J., Pettersson, A., Wilson, D., Mao, Y., Lernmark, A. and Lander, E. S. 1992. Genetic dissection of autoimmune type I diabetes in the BB rat. Nat. Genet. 2:56.
16 Jackson, R., Rassi, N., Crump, T., Haynes, B. and Eisenbarth, G.