Susceptibility and Negative Epistatic Loci Contributing to Type 2 Diabetes and Related Phenotypes in a KK/Ta Mouse Model

Toshihide Shike, Sachiko Hirose, Michimasa Kobayashi, Kazuhiko Funabiki, Toshikazu Shirai, and Yasuhiko Tomino

The KK/Ta mouse strain serves as a suitable polygenic model for human type 2 diabetes. Using 93 microsatellite markers in 208 KK/Ta × (BALB/c × KK/Ta)F1 male backcross mice, we carried out a genome-wide linkage analysis of KK/Ta alleles contributing to type 2 diabetes and related phenotypes, such as obesity and dyslipidemia. We identified three major chromosomal intervals significantly contributing to impaired glucose metabolism: one quantitative trait locus for impaired glucose tolerance on chromosome 6 and two loci for fasting blood glucose levels on chromosomes 12 and 15. The latter two loci appeared to act in a complementary fashion. Two intervals showed significant linkages for serum triglyceride levels, whereas the KK allele on chromosome 4 acts to suppress this effect in a recessive fashion. In addition, it is suggested that the chromosome 4 locus also acts to downregulate body weight and that the chromosome 8 locus acts to upregulate serum insulin levels. Our data clearly showed that each chromosome 4 locus acts to downregulate body weight, whereas the KK allele on chromosome 8 acts to promote serum triglyceride levels, whereas the KK allele on chromosome 4 acts to suppress this effect in a recessive fashion. In the latter suggestion, it is thought that the chromosome 4 locus also acts to upregulate serum insulin levels. Our data clearly showed that each disease phenotype of type 2 diabetes and related disorders in KK/Ta mice is under the control of separate genetic mechanisms. However, there appear to be common genes contributing to different disease phenotypes. There are potentially important candidate genes that may be relevant to the disease. Diabetes 50:1943–1948, 2001

Diabetes is a chronic disorder affecting glucose, fat, and protein metabolism. The disorder is heterogeneous, and several distinct diabetic syndromes have been delineated. Type 2 diabetes, also called non–insulin-dependent diabetes, is a non–autoimmune-mediated disorder characterized by two metabolic defects: a derangement in insulin secretion and an inability of peripheral tissues to respond to insulin (insulin resistance). The manifestations of symptomatic diabetes vary from patient to patient, and a subset of patients presents with glucose intolerance, hyperinsulinemia, dyslipidemia, abdominal obesity, and hypertension, a constellation of symptoms now called the insulin-resistance syndrome (1–4). In patients with premature coronary heart disease, the prevalence of the insulin-resistance syndrome exceeds 40% (5,6). There is reliable evidence that susceptibility to type 2 diabetes and the insulin-resistance syndrome involves multiple genes (7–9). Because of the complexity of inheritance patterns, progress toward establishing these disorders’ molecular basis has been hampered. In this respect, lessons from animal models are invaluable for the analysis of such complex traits. Thus far, inheritance patterns and several chromosomal intervals contributing to type 2 diabetes and the insulin-resistance syndrome have been mapped in several animal models using genome-wide analysis with microsatellite-based chromosomal maps (7–16).

The inbred mouse strain KK/Ta, established in Japan as a diabetic strain, spontaneously exhibits type 2 diabetes associated with fasting hyperglycemia, glucose intolerance, hyperinsulinemia, mild obesity, dyslipidemia, and proteinuria (17–20). Earlier progeny studies by Nakamura and Yamada (21) postulated that the mode of inheritance of glucose tolerance in KK/Ta mice is polygenic. According to Butler and Gerritsen (22,23), using crosses between KK and C57 mice, the inheritance of glucosuria can be explained by assuming that there is a dominant KK gene with 25% penetrance and several recessive modifiers, which may enhance penetrance to 75%. In later studies using microsatellite-based chromosomal maps in the crosses of the congenic strain KK-A^Y/Ta and C57BL/6J mice, Suto and colleagues (10–12) analyzed KK/Ta mouse susceptibility loci contributing to type 2 diabetes and related phenotypes such as dyslipidemia and obesity (adiposity). KK-A^Y/Ta mice are a congenic strain in which the A^Y allele at the agouti locus is introduced into a KK/Ta background by repetitive backcrossing of more than 10 generations, with consequent overt diabetes and massive obesity. Suto and colleagues identified one locus on chromosome 6 for fasting glucose levels, one on chromosome 4 for body weight, one on chromosome 10 for adiposity, and two on chromosomes 1 and 3 for total cholesterol. These data, however, were inconsistent with those found by Taylor et al. (13) using crosses between a substrain KK/HIL4 and C57BL/6J mice. The latter authors identified three KK susceptibility loci: one locus on chromosome 1 for fasting

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ANOVA, analysis of variance; IPGTT, intraperitoneal glucose tolerance test; LCAT, lecithin cholesterol acyltransferase; LOD, logarithm of odds; QTL, quantitative trait locus; PCR, polymerase chain reaction.

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TABLE 1
Mean phenotypic values for parental strains, F1, and backcross mice

<table>
<thead>
<tr>
<th></th>
<th>Ages (weeks)</th>
<th>KK/Ta (n = 11)</th>
<th>BALB/c (n = 11)</th>
<th>(BALB/c × KK/Ta)F1 (n = 18)</th>
<th>KK/Ta × (BALB/c × KK/Ta)F1 backcross (n = 195–208)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>28</td>
<td>44.3 ± 0.8*</td>
<td>29.2 ± 1.3</td>
<td>35.1 ± 1.7†</td>
<td>32.1 ± 0.7</td>
</tr>
<tr>
<td>Fasting blood glucose levels (mg/dl)</td>
<td>12</td>
<td>122.7 ± 5.4†</td>
<td>81.1 ± 3.2</td>
<td>73.6 ± 3.5</td>
<td>93.2 ± 1.6</td>
</tr>
<tr>
<td>Sum of blood glucose at 0 and 120 min (mg/dl)</td>
<td>20</td>
<td>54.5 ± 28.0†</td>
<td>148.5 ± 7.0</td>
<td>195.2 ± 6.0</td>
<td>249.5 ± 2.0</td>
</tr>
<tr>
<td>Sum of insulin levels at 0 and 120 min (mg/dl)</td>
<td>20</td>
<td>5.2 ± 0.8†</td>
<td>1.3 ± 0.3</td>
<td>1.7 ± 0.4</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Serum triglyceride levels (mg/dl)</td>
<td>20</td>
<td>134.8 ± 5.6†</td>
<td>89.0 ± 5.6</td>
<td>69.2 ± 6.5</td>
<td>74.2 ± 1.8</td>
</tr>
<tr>
<td>Serum total cholesterol levels (mg/dl)</td>
<td>20</td>
<td>120.1 ± 4.0</td>
<td>94.0 ± 5.8</td>
<td>100.3 ± 12.1</td>
<td>80.5 ± 1.6</td>
</tr>
</tbody>
</table>

Data expressed as means ± SE. Glucose levels were measured at 0 and 120 min after intraperitoneal glucose injection. Plasma insulin was measured at 0 and 120 min after glucose administration. *Significantly higher than that of BALB/c and F1 (P < 0.0001); †significantly higher than that of BALB/c and F1 (P < 0.001); ‡significantly higher than that of BALB/c (P < 0.0001).

RESULTS
Disease features in KK/Ta, BALB/c, and their crosses.
Phenotypic characteristics of the disorders were examined in male KK/Ta, BALB/c, (BALB/c × KK/Ta)F1, KK/Ta × (BALB/c × KK/Ta)F1 backcross mice. As shown in Table 1, KK/Ta mice displayed obesity, fasting hyperglycemia, impaired glucose tolerance, hyperinsulinemia, and hyperlipidemia compared with the findings in normal control BALB/c mice. The mean body weight of KK/Ta mice was significantly higher than that of BALB/c mice (P < 0.001). The values for F1 mice were intermediate between the parental strains, and the differences between F1 and BALB/c mice were statistically significant (P < 0.0001). Results of fasting blood glucose levels, the sum of blood glucose levels in IPGTT, the sum of plasma insulin levels, and serum triglyceride levels of KK/Ta mice were significantly higher than those of BALB/c and F1 mice (P < 0.001). The differences between F1 and BALB/c mice were not statistically significant. Thus, it appears that genes from the KK/Ta strain determine the disease phenotypes in an incomplete dominant or recessive fashion.

Correlations between disease phenotypes. In KK/Ta × (BALB/c × KK/Ta)F1 backcross mice, there were significant correlations between impaired glucose tolerance and hyperinsulinemia (r = 0.329, P < 0.0001), hypertriglyceridemia and hyperinsulinemia (r = 0.416, P < 0.0001), and hypertriglyceridemia and hypercholesterolemia (r = 0.368, P < 0.0001). Body weight significantly correlated with each of these four phenotypes, suggesting that a certain common genetic mechanism is at least partly involved in

glucose levels, one on chromosome 7 for body weight, and one on chromosome 9 for adiposity, especially in females.

To confirm and extend these studies, we investigated those KK/Ta susceptibility loci contributing to type 2 diabetes with respect to disease phenotypes (such as fasting hyperglycemia, glucose intolerance, and hyperinsulinemia) and the diabetes-related phenotypes, obesity and dyslipidemia. Because expression of some susceptibility genes may be either affected by their alleles or modified by unlinked genes with promoting (enhancing modifier) or even suppressive function (suppressing modifier), we used the BALB/c strain as a crossing partner instead of the C57BL/6 used by other investigators (10–13).

RESEARCH DESIGN AND METHODS
Animals. Inbred mice for this study were purchased from CLEA Japan (Tokyo). BALB/c female mice were mated to KK/Ta males to produce the F1 hybrid mice in our animal facility. KK/Ta × (BALB/c × KK/Ta)F1 backcross mice were obtained by crossing female KK/Ta mice with male (BALB/c × KK/Ta)F1 mice. A total of 208 backcross mice were weaned at age 4 weeks, and from age 6 weeks onward, mice were individually housed in plastic cages with free access to food (rodent pelleted diet CE-2; 342.2 kcal/100 g containing 4.4% crude fat) and water throughout the experimental period. Only male mice were used in the present study. All mice were maintained in the same room under conventional conditions with a regular 12-h light/dark cycle and controlled temperature at 24 ± 1°C.

Phenotypic characterization. The body weight of each mouse was serially monitored at ages 8, 12, 20, 25, and 36 weeks. Glucose tolerance was assessed using the intraperitoneal glucose tolerance test (IPGTT) in mice at ages 12, 20, and 28 weeks. IPGTT was performed by injecting glucose (2 g/kg in 20% solution) intraperitoneally in overnight-fasted mice (24). Glucose levels in blood obtained from the retro-orbital sinus were measured using GluTest E (Kyoto Dainichi Kagaku, Kyoto, Japan) at 0 (fasting blood glucose level) and 120 min after intraperitoneal glucose injection. Impaired glucose tolerance was evaluated on the basis of the sum of blood glucose at 0 and 120 min. In the IPGTT at age 20 weeks, blood was collected using heparinized capillary tubes for measurement of plasma insulin and plasma lipid concentrations (total cholesterol and triglyceride). Plasma insulin was measured by an enzyme-linked immunosorbent assay (Levius insulin kit; Shibayagi, Gunma, Japan) at 0 and 120 min after glucose administration. The degree of hyperinsulinemia was determined by the sum of the plasma insulin levels at 0 and 120 min. Plasma lipid was determined enzymatically with an autoanalyzer (Fuji Dry-Chem 5500; FUJIFILM, Tokyo).

Genotyping. A total of 93 microsatellite marker loci (except for sex chromosomes), polymorphic between KK/Ta and BALB/c, were genotyped in all backcross mice. Genomic DNA was obtained from mouse tails by standard techniques. PCR primers flanking microsatellites were purchased from Research Genetics (Huntsville, AL). Genotyping of KK/Ta × (BALB/c × KK/Ta)F1 backcross progeny for marker loci was performed as described by Dietrich et al. (25). Polymerase chain reactions (PCRs) were run in 96-well plates with 5.0 μl total volume containing 10 ng of genomic DNA. A three-temperature PCR protocol (94, 55, and 72°C) was implemented for 45 cycles in a Geneamp 9700 Thermal Cycler (Perkin-Elmer-Cetus, Norwalk, CT). PCR products were

diagnosed with twofold with loading buffer consisting of xylene cyanol and bromophenol blue dyes in 50% glycerin and were run on 18% polyacrylamide gels. After electrophoresis, gels were visualized after ethidium bromide staining.

Linkage and statistical analyses. Linkage analysis was performed using both Pearson’s χ² test and interval mapping. Genomic interval mapping was conducted using the Map Manager QT program package originally described by Manly (26). P values <0.05 were considered statistically significant. To estimate positions of quantitative trait loci (QTLs), the likelihood ratio statistics were determined using the Map Manager QT program package. Logarithm of odds (LOD) scores of ≥1.9 and ≥3.3 were used as thresholds for statistically suggestive and significant linkage, respectively, according to Lander and Kruglyak (27). In addition to the criteria using the fixed level of LOD, the permutation test was also performed in 1-cM steps for 500 permutations using the Map Manager QT program package to further estimate significance levels for QTL analysis (26). Correlations between the different parameters were analyzed by linear regression with StatView 4.0 on a Macintosh. Analysis of variance (ANOVA) was used to determine differences in the extent of disease characterizations among each group of backcross progeny with different combinations of susceptibility alleles.
the regulation of these phenotypes. In contrast, these phenotypes were not significantly associated with fasting blood glucose levels.

**Mapping of susceptibility alleles.** To determine the genetic basis of each disease phenotype, we performed mapping of susceptibility alleles using microsatellite markers. Mapping of KK/Ta-derived genes contributing to each disease phenotype entailed genotyping 208 male KK/Ta × (BALB/c × KK/Ta)F1 backcross mice with 93 microsatellite markers. The average marker distance was 14.1 cM, and the genome coverage estimated by the percentage of each chromosome within 20 cM of the marker loci was 90%. The association between the positive phenotype and the genotype of the microsatellite marker locus (KK/KK homozygous or KK/BALB/c heterozygous type) was examined; data from QTL analyses at a given marker locus in the backcross mice are shown in Fig. 1.

The marker loci distributed in chromosomes 3, 4, 6, 8, 12, and 15 showed either significant or suggestive linkage with one of the six disease phenotypes examined. Among these, three KK loci were linked to impaired glucose metabolism. As shown in Fig. 1A, two loci on chromosomes 12 and 15 showed either significant or suggestive linkage with one of the six disease phenotypes examined. Among these, three KK loci were linked to impaired glucose metabolism. These data are consistent with the idea that this KK locus acts to suppress serum triglyceride levels in a recessive fashion; thus, we designated this locus *Tgs-1* (serum triglyceride level suppressor-1), with a peak LOD of 4.0 (χ² = 11.7, P = 0.0008) was found in mice at age 20 weeks on chromosome 6 near marker *D6Mit1*, which accounted for 11% of the phenotypic variation (direction: KK/KK > KK/BALB).

Serum triglyceride levels showed linkages with two intervals on chromosomes 4 and 8 (Fig. 1B). One locus mapped on chromosome 8 of KK mice, designated *Tg-1* (serum triglyceride level-1), was identified between *D8Mit242* and *D8Mit166*, with a maximum LOD of 4.8 (χ² = 14.3, P = 0.0002), and explained 13% of the phenotypic variance (direction: KK/KK > KK/BALB). On the other hand, the second locus, mapped in the proximity of *D4Mit336*, showed a suggestive linkage with a maximum LOD of 3.2 (χ² = 4.8, P = 0.02), in that the KK/BALB heterozygotes had significantly higher serum triglyceride levels than did the KK/KK homozygotes (direction: KK/BALB > KK/KK). To further estimate the significance level of this linkage, the permutation test was also performed. The result showed that the likelihood ratio statistic value was 14.7, higher than the critical value of significant linkage (11.1), indicating that the linkage of serum triglyceride levels with *D4Mit336* was significant. These data are consistent with the idea that this KK locus acts to suppress serum triglyceride levels in a recessive fashion; thus, we designated this locus *Tgs-1* (serum triglyceride level suppressor-1). *Tgs-1* explained 4% of the phenotypic variance. It was intriguing that *Tg-1* also showed a suggestive linkage to hyperinsulinemia, with an LOD of 2.2 (direction: KK/KK > KK/BALB), and *Tgs-1* showed a suggestive linkage to body weight, with an LOD of 2.1 (direction: KK/BALB > KK/KK). Permutation tests of these two linkages also showed a suggestive linkage. A single dominant KK locus, designated *Igt-1* (impaired glucose tolerance-1), with a peak LOD of 8.27, P = 0.0004), was on the middle portion of chromosome 12 between *D12Mit4* and *D12Mit227* and accounted for 11.8% of the phenotypic variation. A single KK locus, designated *Fbg-1* and *Fbg-2* (fasting blood glucose level-1 and -2), respectively. *Fbg-1*, with a peak LOD of 4.5 (χ² = 8.27, P = 0.0004), was on the middle portion of chromosome 12 between *D12Mit4* and *D12Mit227* and accounted for 11.8% of the phenotypic variation. *Fbg-2*, with a peak LOD of 3.3 (χ² = 5.8, P = 0.016), was identified on the centromeric portion of chromosome 15 near *D15Mit225*, and accounted for 6.0% of the phenotypic variation. A single KK locus, designated

![Image](https://example.com/image.png)

*FIG. 1. QTL analysis of impaired glucose metabolism (A) and other type 2 diabetes-related phenotypes (B) in KK/Ta × (BALB/c × KK/Ta)F1 backcross progeny at a given week of age in each phenotype (see Table 1). LOD score curves are shown along chromosomes. Mapping positions of the typed microsatellite markers are indicated on the left with the Mouse Chromosome Committee cM distances from the centromere in parentheses. The loci of potential candidate genes are shown on the right of each chromosome. Each phenotype is boxed: IGT, impaired glucose tolerance; FBS, fasting blood glucose levels; TCHOL, hypercholesterolemia; BW, body weight; TG, hypertriglyceridemia; HI, hyperinsulinemia. The number on the top of the vertical line represents the LOD score.*

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nated Tcho-1 (serum total cholesterol level-1), was mapped on chromosome 3 near the marker D3Mit12, with a peak LOD of 4.0 ($\chi^2 = 17.7$, $P < 0.0001$) (direction: KK/KK > KK/BALB). Tcho-1 accounted for 11% of the phenotypic variance.

Gene interactions. Because two unlinked loci in the vicinities of D12Mit14 and D15Mit225 on the one hand and D4Mit336 and D8Mit166 on the other showed linkages to fasting blood glucose levels and hypertriglyceridemia, respectively, we assessed their genetic interactions with these two loci. The backcross mice were separated into four groups (A–D) according to combined genotyping of loci D12Mit14 and D15Mit225 (A) and D4Mit336 and D8Mit166 (B). Levels of fasting blood glucose at age 12 weeks and serum triglycerides at age 20 weeks are expressed by means ± SE. KK/KK homozygote for KK/Ta allele; KK/BALB, heterozygote for KK/Ta and BALB/c allele. *$P < 0.005$; **$P < 0.01$; ***$P < 0.001$. Data of parental and F1 strains are also included.

**DISCUSSION**

Our data, using crosses between mouse strains KK/Ta and BALB/c, clearly showed the polygenic pattern of diabetic inheritance in KK/Ta mice. We identified several susceptibility loci contributing to type 2 diabetes and related phenotypes, such as dyslipidemia and obesity. As summarized in Table 2, to our surprise, the results of our QTL analyses were not consistent with those reported by other investigators (10–13), except for one locus for serum total cholesterol levels mapped on chromosome 3 by Suto et al. (12). The lack of concordance among these studies might be related to a number of differences, including ages at the time of phenotype evaluation, sample size, methods for measurement of phenotypes, crossing partners (C57BL/6J vs. BALB/c), and potential KK substrain differences.

In our studies, several loci responsible for glucose metabolism were independently distributed on different chromosomes. Thus, fasting glucose levels and postprandial glucose levels were suggested to be at least partly under separate genetic control. We identified two KK/Ta susceptibility loci for fasting blood glucose levels on chromosomes 12 (Fbg-1) and 15 (Fbg-2), which were considered to act in a complementary manner (Fig. 2A). The interval closely linked to Fbg-1 contains the gene-encoding liver glycogen phosphorylase ($Pyg-1$) (Fig. 1) (28). A balance between hepatic glucose production and glucose uptake maintains fasting plasma glucose concentration in peripheral tissues. $Pyg-1$ regulates hepatic glucose production; hence, a potential polymorphism of $Pyg-1$ may lead to a high fasting plasma glucose concentration that exceeds the extent of glucose uptake. Another candidate gene of interest in this region is the gene for thyroid-stimulating hormone receptor (Tshr). In this respect, it is noteworthy that the interval closely linked to $Fbg-2$ contains the gene encoding growth hormone receptor ($Ghr$). A combined effect of insulin and glucagon primarily provides hormonal regulation of hepatic glucose production. Because both thyroid-stimulating hormone and growth hormone influence insulin action, it is suggested that abnormalities of these hormone receptors are responsible for fasting blood glucose levels. Warden et al. (29) reported two obesity-related loci on chromosomes 12 (Mob-3) and 15 (Mob-4) using crosses between *Mus musculus* and C57BL/6J mouse strains. These loci are located near $Fbg-1$ and $Fbg-2$, respectively. In the present studies,
however, there were no significant linkages between these intervals and body weight.

The QTL for Igt-1 was detected on chromosome 6 near D6Mit1. According to Warden et al. (29,30), the locus linked to this marker locus has been found to affect fat accumulation (designated Mob-2) and serum total cholesterol levels (designated Chol-2) and has a suggestive linkage for serum insulin levels. In the present studies, we did not perform the linkage analysis for adiposity. However, there were no significant linkages of this interval with body weight, serum total cholesterol levels, or serum insulin levels. Therefore, Igt-1 may not correspond to Mob-2 and Chol-2.

Two chromosomal intervals on chromosomes 4 (Tgs-1) and 8 (Tg-1) of KK/Ta mice were found to affect hypertriglyceridemia. Tg-1 acts to promote serum triglyceride levels in a recessive fashion, whereas this effect is suppressed by a recessive Tgs-1. Intriguingly, it is suggested that Tgs-1 also acts to downregulate body weight, and Tg-1 acts to upregulate the serum insulin level. Although the linkages were suggestive, these findings are interesting because hypertriglyceridemia, obesity, and hyperinsulinemia are thought to be major clinical features in the human insulin-resistance syndrome (1–4).

One of the possible candidate genes in the Tg-1 region is the gene Lcat, encoding lecithin cholesterol acyltransferase (LCAT) (31). LCAT is a glycoprotein synthesized primarily by the liver that plays a major role in the metabolism of HDL (32). Sakai et al. (33) have established a mouse model for human LCAT deficiency by targeted disruption of Lcat in mouse embryonic stem cells. These authors reported that serum triglyceride levels were significantly higher in homozygous knockout male mice than in wild-type animals. Based on this information and our mapping data, Lcat can be considered as the most plausible candidate gene for Tg-1 in KK/Ta mice.

This study identified Tcho-1 for serum total cholesterol levels on chromosome 3 in KK/Ta mice. This locus is very close to the hyperlipidemia gene (Hyplip-J), contributing to hypertriglyceridemia and hypercholesterolemia in a set of recombinant inbred strains derived from C3H/DisNa and C57BL/10ScSnA strains (15). Suto et al. (12) obtained evidence suggesting that there are two QTLs affecting total cholesterol levels on chromosomes 1 and 3 using (C57BL/6J × KKAY)F2 mice. The locus on chromosome 3 was located near the marker D3Mit102. Because this locus is very close to the position of D3Mit12, this gene may be identical to Tcho-1 in the present studies. This region is syntenic to human chromosome 1q21-q23, which has been shown to harbor a gene associated with familial combined hyperlipidemia in families from a Finnish isolate (34).

In conclusion, we identified several new loci contributing to type 2 diabetes and related phenotypes. One KK locus was found to act to suppress serum triglyceride levels in a recessive fashion. The recessive effect of this gene is reminiscent of a tumor-suppression gene whose tumorigenic phenotype is recessive (35). Genetic analysis of the corresponding chromosomal regions in humans, and examination of candidate genes residing at the loci, may provide a more thorough understanding of genetic factors involved in human type 2 diabetes and the insulin-resistance syndrome. On the basis of such analysis, prophylactic and therapeutic clinical approaches can be designed.

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**REFERENCES**


