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Virus Persists in β Cells of Islets of Langerhans and Is Associated with Chemical Manifestations of Diabetes

Abstract. *Molecular hybridization, monoclonal antibody, and electron microscopic analyses showed lymphocytic choriomeningitis virus (strains Armstrong and WE) persistently infecting cells of the islets of Langerhans in BALB/WEHI mice. When monoclonal or monospecific antibody conjugated with two different fluorochrome dyes was used to mark insulin-containing β cells or viral antigens, viral nucleoprotein was identified predominately in β cells. Electron microscopy confirmed these findings by showing virions budding from the β cells. Persistent infection was associated with chemical evidence of diabetes (hyperglycemia, abnormal glucose tolerance, and normal or low-normal concentrations of insulin). Concentrations of cortisol and insulin-like growth factor in blood were normal, as was the level of growth hormone in the pituitary gland. The virus-infected islet cells showed normal anatomy and cytomorphology. Neither cell lysis nor inflammatory infiltrates were routinely seen. Thus a virus may persistently infect islet cells and provide a biochemical and morphological picture comparable to that of early adult-onset diabetes mellitus in humans.*

Diabetes mellitus, one of the most common metabolic disorders in humans, may be caused by a deficiency of insulin in genetically predisposed individuals (*1*) or may result from increased resistance of such individuals to the action of insulin (*1*, *2*). The deficiency of insulin, at least in juvenile-onset diabetes, may result from destruction of β cells in the islets of Langerhans by virus or by autoimmune constituents. Suspicion that viruses cause some cases of juvenile-onset diabetes originated from epidemiologic and pathologic surveys of humans and from animal studies (*3*). Recently, Yoon *et al.* (*4*) provided direct evidence for a viral etiology by isolating and identifying Coxsackie B4 virus from the pancreas of a child with acute diabetes. Transfer of this isolate into mice led to injury of islet cells and hyperglycemia.

The cause of diabetes beginning in adulthood is more obscure than that of juvenile diabetes. One theory implicates faulty nutrition. Because viral infection may cause juvenile-onset diabetes by destroying islet β cells, we sought to determine whether a virus can persist in the β cells of adults, perhaps altering the synthesis or function of insulin. We found that a virus, lymphocytic choriomeningitis virus (LCMV), can indeed persist in β cells of adults and be associated with aberrations in blood chemistry similar to those found in the early stages of adult-onset diabetes.

Newborn BALB/WEHI mice were inoculated intracerebrally with 60 plaque-forming units (PFU) of LCMV strain WE (30 mice) or LCMV Armstrong strain 1371 (30 mice), each strain having been cloned three times. Over 80 percent of the mice were alive 90 days later and all were persistently infected with LCMV in their blood and organs (5×10^3 to 5×10^5 PFU per milliliter), despite mounting antiviral immune responses (*5*).

Three methods were used to determine whether LCMV persisted in islet cells: molecular hybridization to localize LCMV nucleic acid, immunochemical staining to identify specific viral polypeptides, and electron microscopy to characterize virions. LCMV is a negative strand virus containing two RNA segments designated L and S, with approximate lengths of 9 and 4 kilobases (kb), respectively (*5*, *6*). The viral genome codes for three known structural polypeptides (*7*). Two of these are glycosylated and are designated GP1 and GP2 (molecular weights, 43,000 and 36,000, respectively). The third viral polypeptide is not glycosylated and constitutes the nucleoprotein (molecular weight, 63,000). GP1 and GP2 are expressed on the surfaces of infected cells or virions, being derived in equimolar amounts from a common precursor polypeptide, GPC (*7*), that is also expressed on the cell surface. A fourth suspected polypeptide, the polymerase, has not been thoroughly characterized.

The L and S RNA segments of LCMV Armstrong 1371 were isolated, reverse-transcribed into complementary DNA (cDNA), and cloned into pBR322 vector (*8*). When labeled with ^{32}P by nick translation (*8*, *9*), these cDNA probes hybridized specifically to LCMV Armstrong 1371 L or S RNA extracted from virions

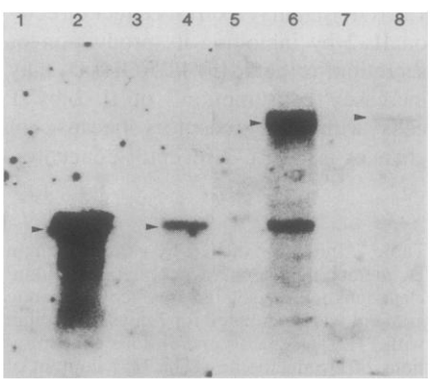

Fig. 1. Demonstration of LCMV L and S RNA segments in islet cells from 90-day-old BALB/WEHI mice persistently infected with LCMV Armstrong strain 1371 (clone 3b). When less than 18 hours old, these mice received 60 PFU of virus injected intracerebrally. At 90 days of age all the mice carried infectious virus, as determined by PFU assay or by intracerebral transfer of their serum into susceptible mice 4 to 6 weeks old. Pancreases from ten infected and ten uninfected mice were pooled separately, minced, treated with collagenase (5 mg/ml), and dissolved in Hanks balanced salt solution. After gentle stirring for 30 minutes at 37°C, dissociated cells obtained by low-speed centrifugation were placed on a discontinuous 40 percent gradient (*10*). Islet cells remained in the upper fraction and were recognized by their morphology and ability to secrete insulin in vitro and by their insulin content. RNA from the islet cells and from hybridoma 1-1-3 was obtained after treatment with guanidinium thiocyanate and analyzed by Northern blots for LCMV Armstrong 1371–specific sequences by using cDNA probes for LCMV Armstrong RNA segments L and S. Such probes do not bind to RNA extracted from uninfected cells or from cells infected with LCMV WE. Shown are sequential hybridization reactions with the same nitrocellulose filter. A cDNA probe for LCMV RNA segment S was used first (lanes 1 to 4), then a probe for segment L (lanes 5 to 8). Lanes 1 and 5 represent uninfected 1-1-3 cells; lanes 2 and 6, LCMV Armstrong–infected 1-1-3 cells; lanes 3 and 7, LCMV WE–infected islet cells; and lanes 4 and 8, LCMV Armstrong–infected islet cells. The bands in lanes 2 and 4 (arrows) represent genome-sized S RNA; the bands in lanes 6 and 8 (arrows), genome-sized L RNA. Under these conditions of hybridization this L probe shows some homology to S-sized RNA.

or infected BHK or L-929 cells, but showed no hybridization to RNA extracted from uninfected BHK or L-929 cells or to cells infected with LCMV WE. Similarly, these probes specifically hybridized to RNA obtained from pancreas or brain tissue from 90-day-old mice persistently infected with LCMV Armstrong, demonstrating the presence of LCMV L and S RNA in these tissues. In contrast, such probes failed to bind to RNA from pancreas or brain tissues from uninfected mice. Because LCMV infects several pancreatic cell types in addition to islet cells, we enriched the islet cell population on discontinuous 40 percent gradients (Ficoll) and monitored them morphologically with a phase-contrast microscope and biochemically by analyzing for insulin content (*10*). RNA extracted from the enriched islet cell population [> 85 percent pure by the above criteria and by immunofluorescence detection of insulin-containing cells (*10*)] from LCMV-infected mice contained LCMV L and S segments (Fig. 1), but there was no detectable hybridization to RNA in the population from uninfected mice.

Using appropriate monoclonal antibodies to LCMV polypeptides (*11*), we found LCMV nucleoprotein expressed in islet cells from all 60 infected mice (Fig. 2B). On the average, 70 percent of cells from each islet contained viral nucleoprotein (range, 15 to 95 percent). In contrast, LCMV glycoproteins were observed in islet cells from only three infected mice. Restricted expression of LCMV glycoprotein in the presence of abundant LCMV nucleoprotein has been noted before in cultures of persistently infected BHK, L-929, and neuroblastoma cells and in brain, pituitary gland, kidney, and liver cells from persistently infected mice (*11, 12*). Use of mouse monoclonal antibody to LCMV nucleoprotein and of guinea pig monospecific antibody to insulin conjugated to two different fluorochrome dyes (*11*) on the same cryostat section of pancreas indicated that insulin-containing β cells were persistently infected. Most of the infected cells in the islet were β cells; however, several non–insulin-bearing cells in the islet also contained viral gene products. Similar results were obtained when islet cells from ten other BALB/WEHI mice infected with LCMV for 6 months or more were studied with the same reagents.

Electron microscopic study of over 50 islets obtained from 15 BALB/WEHI mice infected with LCMV WE for 3 months showed that an average of 2 percent (range, 1 to 5 percent) of islet cells contained budding virions. The virions budded predominately from β cells (Fig. 2D), but also from α cells (*13*). Although the cells replicated LCMV, necrosis and inflammatory responses were observed in less than 5 percent of more than 500 islets studied by light microscopy from 50 mice, and the islets and islet cells remained structurally normal (Fig. 2A). However, islets from persistently infected mice appeared larger than islets from age- to sex-matched controls. Quantitative analysis indicated that the enlargement of islets was due to an increase in the size of individual cells and not to an increase in the number of cells (*14*). Such hypertrophy of islet cells may result from an increase in the de-

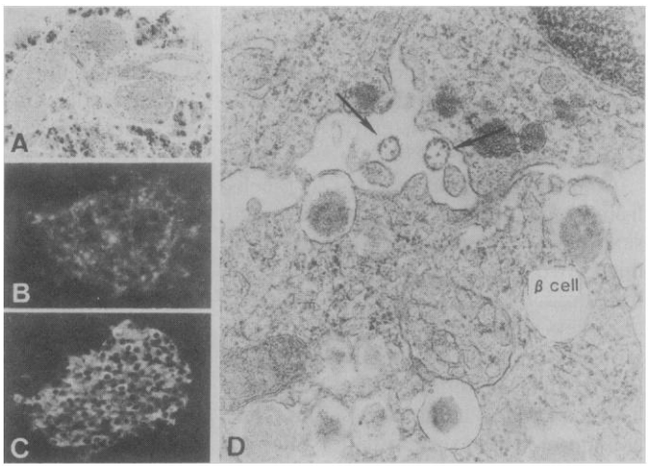

Fig. 2. Islets of Langerhans from 90-day-old BALB/WEHI mice persistently infected with LCMV WE (clone c). Infection was initiated and tested as described in the legend to Fig. 1. (A) Low-power (×250) photomicrograph of islets. Cytomorphology was normal and no cell lysis or inflammatory infiltrates were observed. (B) Islet cells containing LCMV antigens (×500). Cryostat sections (4 μm) of pancreas were stained with mouse monoclonal antibody to LCMV nucleoprotein and goat antibody to mouse IgG conjugated to rhodamine (*11*). Results were similar in over 50 islets from individual LCMV-infected mice examined with this reagent or with a guinea pig polyclonal antibody to LCMV conjugated to fluorescein isothiocyanate. Neither reagent stained islet cells or other cells from uninfected mice. The β cells in the islet were identified by labeling insulin-containing cells with monoclonal or polyclonal antibody to insulin and a different fluorochrome dye (*11*), as shown in (C) (×500). (D) Electron micrograph (×40,000) showing LCMV virions (arrows) in β cells. The cells have a characteristic morphology, including amorphous granules and a clear halo between the granules and the membranous vesicles that contain them (*13*). Similar results were obtained with β cells in islets from 90-day-old BALB/WEHI mice infected with LCMV Armstrong or from 90-day-old SWR/J mice infected with LCMV Armstrong or WE.

Table 1. Perturbation of glucose metabolism associated with persistent LCMV infection in β cells from 90-day-old BALB/WEHI mice. Within 18 hours of birth, the mice were inoculated with 60 PFU of triple-cloned LCMV WE (the clone used was designated c) or triple-cloned LCMV Armstrong strain 1371 (the clone used was designated 3b). Samples from individual mice were assayed. Glucose concentration was determined in 5 to 20 μl of serum by using a microautomatic Beckman Astra-8 analyzer. Glucose tolerance was tested by injecting each mouse with glucose (2 mg/kg, intraperitoneally) and comparing blood samples obtained 1 hour later with those drawn before the glucose challenge. A twofold or greater increase over the baseline value was considered abnormal (*16*). Cortisol and insulin concentrations were determined by radioimmunoassay and a competitive inhibition technique. Insulin-like growth factor was measured by radioimmunoassay (*17*). Pituitary growth hormone was isolated with acrylamide gel electrophoresis and measured by radioimmunoassay (*18*). Values are means ± standard errors, except those for abnormal glucose tolerance, which are numbers of mice.

Assay	Minimum number of mice per group	Uninfected mice	LCMV-infected mice	
			WE c	Armstrong 1371 3b
Blood glucose (mg/dl)				
Experiment 1	21	121 ± 12	164 ± 8*	158 ± 5*
Experiment 2	10	97 ± 6	207 ± 12*	164 ± 7*
Abnormal glucose tolerance		2 of 41	19 of 25*	15 of 30*
Blood insulin (μU/ml)				
Experiment 1	10	24 ± 5	31 ± 4	25 ± 4
Experiment 2	15	43 ± 7	29 ± 4	46 ± 11
Blood cortisol (ng/ml)	10	11 ± 1	9 ± 1	10 ± 1
Blood insulin-like growth factor (U/ml)	10	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
Growth hormone (μg/mg pituitary gland)	7	6 ± 2	9 ± 2	5 ± 2
Body weight (g)	44	25 ± 1	22 ± 1	24 ± 1

*Significantly different from corresponding control value ($P < 0.01$, Student's t-test).

mand for insulin. The lack of necrosis is probably related to the noncytopathic nature of LCMV. The absence of inflammatory cells in the immediate environment of these infected cells may have resulted from the low levels of viral glycoprotein expressed and the apparent absence of budding virions. It is likely that viral glycoprotein must appear on the surface of infected cells in an amount sufficient to incite recognition and thereby allow assault by the host's immune effector system. Another factor is the decrease in LCMV-specific T-lymphocyte help and cytotoxic T-lymphocyte responses in persistently infected BALB/WEHI mice (*15*).

Amounts of insulin secreted by β cells from LCMV-infected and uninfected sex- and age-matched BALB/WEHI mice (two mice per group) were then measured. Pancreases were removed and islets were recovered through Ficoll gradients and plated (50 per petri dish) in RPMI 1640 medium with 10 percent heat-inactivated fetal calf serum. The amount of insulin secreted after 24 hours in culture was measured by radioimmunoassay (*10*). In three separate experiments, islets from mice infected with LCMV WE and Armstrong secreted significantly more insulin (37 ± 6 and 76 ± 9 μU, respectively) than did islets from uninfected mice (17 ± 3 μU) [$P < 0.02$ and $P < 0.001$, respectively (Student's t-test)]. Thus, production of insulin by β cells harvested from persistently infected mice increased, possibly as a response to elevated blood glucose concentrations and perhaps reflecting islet cell hypertrophy.

Because immunofluorescence and electron microscopy indicated LCMV persisting in cells of the islets of Langerhans, we studied the effect of this infection on cell function in vivo, that is, regulation of glucose metabolism. As Table 1 illustrates, 90 days after the initiation of LCMV infections BALB/WEHI mice had significantly higher blood glucose concentrations than matched uninfected control mice. Of 25 LCMV WE–infected BALB/WEHI mice given glucose (2 mg/kg, intraperitoneally) and tested before and 1 hour after challenge, 19 (76 percent) showed abnormal glucose tolerance (Fig. 3). In contrast, only 2 of 41 age- and sex-matched uninfected controls showed a corresponding abnormality. Mice persistently infected with LCMV Armstrong were intermediate in their response to glucose, with 15 of 30 showing perturbations. In contrast, the concentrations of blood insulin, cortisol, insulin-like growth factor (a measure of growth hormone activity), and pituitary growth hormone were within normal limits (Table 1).

These findings show that a noncytopathic virus, LCMV, is tropic for cells in the islets of Langerhans and can establish a persistent infection there. In vivo, the virus persists in such cells without killing them and without attracting immune reactants. Nevertheless, persistent infection is associated with hyperglycemia and abnormal glucose tolerance. Thus, a virus can persistently infect islet cells, including those that make insulin. The end result is a chemical and morphological picture similar to that observed in early stages of adult-onset diabetes mellitus. Whether this phenomenon occurs in humans remains to be determined. Also, it is necessary to ascertain (i) whether mice persistently infected with LCMV and made obese or having subclinical impairment of islet cells caused by other environmental factors develop clinical manifestations of diabetes, (ii) the effect of virus infection on non–β cells in the islets, and (iii) how LCMV infection may affect expression of the insulin gene.

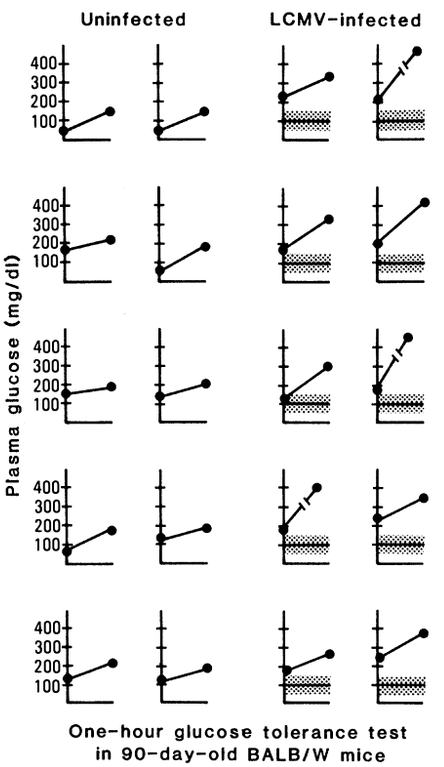

Fig. 3. Perturbation of glucose metabolism in 90-day-old BALB/WEHI mice persistently infected with LCMV WE (clone c). None of the ten uninfected mice represented developed twofold increases in blood glucose 1 or 2 hours after receiving glucose (2 mg/kg). (Similar results were obtained for 29 of 31 other uninfected mice.) In contrast, eight of the ten LCMV-infected mice represented had far greater than twofold enhancement of blood glucose under similar conditions. This abnormality was found in 19 of 25 LCMV WE–infected mice studied. The heavy horizontal lines and shaded areas indicate means ± 2 standard errors for uninfected mice.

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Acetolactate Synthase Is the Site of Action of Two Sulfonylurea Herbicides in Higher Plants

Abstract. *Biochemical and genetic studies of a tobacco mutant resistant to the herbicides chlorsulfuron and sulfometuron methyl have demonstrated that these sulfonylurea herbicides inhibit acetolactate synthase, the first enzyme specific to the branched chain amino acid biosynthetic pathway. Resistance of this mutant is accomplished by production of a form of the enzyme that is insensitive to inhibition by the two herbicides.*

Chlorsulfuron and sulfometuron methyl are the active ingredients in the two herbicides, Glean and Oust (DuPont), respectively. *Nicotiana tabacum* mutants resistant to these compounds have been isolated from cell cultures. One such mutation, *S4*, increased resistance of plants to chlorsulfuron at least 100-fold when present in the homozygous state (*1*). Biochemical characterization of mutant plants has now revealed both the site of action of these herbicides and the mechanism by which resistance is effected.

The discovery that sulfometuron methyl inhibits growth of *Salmonella typhimurium* in the presence of valine and that this inhibition is reversed specifically by isoleucine first suggested that this herbicide acts by interfering with an early step in the biosynthesis of the branched-chain amino acids (*2*). Similar patterns of reversal of growth inhibition were obtained with pea (*Pisum sativum*) and the yeast *Saccharomyces cerevisiae*. Growth inhibition by chlorsulfuron of pea seedlings and root cultures was prevented by supplementation of the medium with isoleucine and valine (*3*). Growth of yeast in the presence of sulfometuron methyl was restored by addition of isoleucine, leucine, and valine (*4*). Direct assay of the first enzyme specific to the isoleucine-leucine-valine biosynthetic pathway, acetolactate synthase (ALS) (E.C. 4.1.3.18), demonstrated the sensitivity of the ALS activities of these three organisms (*2–4*) and of tobacco (Fig. 1) to extremely low concentrations of chlorsulfuron and sulfometuron methyl.

However, demonstration of the sensitivity of an enzyme to a herbicide is necessary, but not sufficient, evidence to identify the site of action of that herbicide. A possibility that is too often overlooked in such investigations is that the herbicide inhibits other enzymes in addition to the one identified (perhaps within the same metabolic pathway) and that it is primarily the action of the herbicide on one of these other activities that determines the response of the whole organism.

A particular enzyme can be established as the primary (or sole) site of action of a herbicide by a combination of genetic and biochemical lines of evidence that include demonstration that mutants resistant to a herbicide possess an altered form of an enzyme that is insensitive to the herbicide and that the resistant form of the enzyme cosegregates with the resistance phenotype in genetic crosses. We now report the results of such experiments, which provide strong evidence that ALS is the site of action of the sulfonylurea herbicides chlorsulfuron and sulfometuron methyl in higher plants.

A plant heterozygous for the *S4* mutation was constructed by a series of backcrosses of a plant regenerated from a herbicide-resistant tobacco cell line with plants of the parental variety (*N. tabacum* cv. Xanthi). Self-fertilization of this heterozygous individual yielded homozygous mutant, heterozygous, and ho-

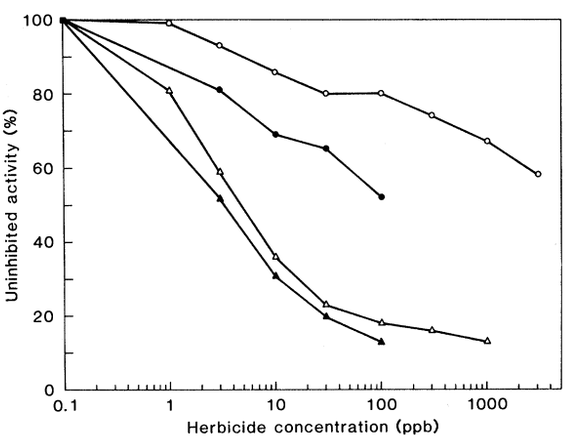

Fig. 1. Responses to chlorsulfuron and sulfometuron methyl of ALS activities in extracts of normal (H1) and homozygous mutant (Isolate 16) cell lines. Cell suspension cultures were washed in liquid medium (*6*) and extracted in a 100 m*M* potassium phosphate buffer (*p*H 7.5) containing 0.5 m*M* $MgCl_2$, 1 m*M* sodium pyruvate, 0.5 m*M* thiamine pyrophosphate, 10 μ*M* flavine-adenine dinucleotide (FAD), and 10 percent (by volume) glycerol. Polyclar AT (BDH Chemicals Ltd.) was added to a final concentration of 250 mg per gram fresh weight of tissue. After centrifugation at 27,000*g* for 15 minutes at 4°C, an equal volume of a saturated solution of ammonium sulfate was added to the supernatant fraction. The resultant precipitate (representing a 0 to 50 percent ammonium sulfate fraction) was redissolved in 100 m*M* potassium phosphate buffer (*p*H 7.5) containing 0.5 m*M* $MgCl_2$ and 1 m*M* sodium pyruvate, and salts were removed by passage through a Sephadex G-25 (Pharmacia PD-10) column. ALS activity was assayed in a total volume of 500 μl containing 100 μl of the column eluate, 65 m*M* potassium phosphate buffer (*p*H 7.5), 40 m*M* sodium pyruvate, 10 m*M* $MgCl_2$, 250 μ*M* thiamine pyrophosphate, 23 μ*M* FAD, and the indicated concentration of chlorsulfuron or sulfometuron methyl. After incubation at 30°C for 90 minutes, 250 μl of 6*N* H_2SO_4 was added to each assay tube, and the tubes were incubated at 55°C for 10 minutes to terminate the enzymatic reaction and achieve complete conversion of acetolactate to acetoin. Acetoin content was then measured by a modification of the procedure described (*14*). To each tube were added sequentially 100 μl of 50 percent NaOH, 150 μl of 0.5 percent creatine, and 150 μl of 5 percent α-naphthol (in 2.5*N* NaOH). Color development was accelerated by incubation at 55°C for 10 minutes. Tubes were centrifuged, and the optical density was read at 530 nm. Background optical densities, which were subtracted from the optical density values of the reactions, were determined for each extract from parallel assays to which the enzyme extract was added after the addition of 6*N* H_2SO_4. Protein concentrations were measured with Bio-Rad Protein Assay reagent with bovine serum albumin as a standard. Data points and specific activities (see text) are the average values of at least three independent determinations. ALS activities are presented as percent activity in the absence of herbicide. (▲) H1 activity in the presence of sulfometuron methyl, (△) H1 activity in the presence of chlorsulfuron, (●) *S4*/*S4* mutant activity in the presence of sulfometuron methyl, and (○) *S4*/*S4* mutant activity in the presence of chlorsulfuron.