differ in only a few nucleotides[13]. Future work will therefore attempt to disarm the satellite such that it is no longer capable of transmission from the transformed plant.

An alternative strategy for transformation of plants to enhance their virus resistance has been described in which the viral coat protein is produced in the transformed plants. This approach, which is an attempt to simulate natural cross-protection between virus strains[14,15]; seems applicable to a wide range of viruses. However, it differs from the approach we have adopted both in its concept and its effects. Most importantly, the coat protein strategy results primarily in symptom retardation and is sensitive both to the strength of the viral inoculum (in the range from 0.4 to 2.0 $\mu g\ ml^{-1}$ for tobacco mosaic virus) and to the amount of coat protein expressed[14]. This, presumably, is because the effect of the expressed coat protein is overcome when the viral RNA accumulates above a certain concentration. Satellite RNA, in contrast, is a molecule that is amplifiable by the incoming virus. Thus, although the system does not give complete inhibition of virus replication, and indeed requires the virus to replicate for the satellite to be replicated, the protection is permanent, independent of the strength of the viral inoculum, and insensitive to the concentration of satellite transcript in the transformed plants. Further work is required to explore whether this strategy of transformation with satellite can be applied to other plant viruses.

We thank Joanne Clark, Anne Jolly, Elizabeth Murant and Graham Saunders for technical help. The work was supported in part by the Rockefeller Foundation and the Agricultural Genetics Company Ltd.

Received 27 May; accepted 29 June 1987.

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Construction of a plant disease resistance gene from the satellite RNA of tobacco ringspot virus

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Tobacco ringspot virus (TobRV) is the type member of the nepoviruses[1]. It consists of 28-nm isometric particles which contain one or the other of the two single-strand genomic RNAs of 4.8 and 7.2 kilobases (kb) (refs 2 and 3). TobRV infects a wide range of dicotyledonous plants and is the causative agent of the budblight disease of soybean. A small RNA which can replicate to high levels and be encapsidated by TobRV in infected plants has been found during serial passages of virus isolates[4,5]. It is not required for virus propagation and has no detectable sequence homology with the virus genomic RNAs. It is therefore termed the satellite RNA of tobacco ringspot virus (STobRV). It can be considered a parasite of the virus and it ameliorates disease symptoms when present during virus infection of plants. We report here the expression of forms of the STobRV sequence in transgenic tobacco plants. Plants which express full-length STobRV or its complementary sequence as RNA transcripts show phenotypic resistance when infected with TobRV. This is correlated with the amplification of satellite RNA to high levels during virus infection of plants.

The complete nucleotide sequence of the budblight isolate of STobRV is known[6], and does not appear to encode functional polypeptide products. As is conventional, we will refer to the polarity of the packaged RNA strand as (+), and that of the complementary strand as (−). STobRV replicates through (−)strand intermediates[7], in an apparently similar way to other related circular satellite RNAs (refs 8 and 9), and 'rolling circle' mechanisms have been proposed to account for the concatameric (+) and (−)strand transcripts found in infected tissues[7]. Both (+) and (−)strand STobRV concatamers undergo self-catalysed ribonucleolytic cleavage at unique sites *in vitro*[10-12], and may do so *in vivo*, to produce unit-length STobRV sequences. Inoculation of TobRV infected plants with autolytically-cleaved *in vitro* transcripts of (+)STobRV resulted in the propagation of STobRV and amelioration of virus induced symptoms[13]. In contrast, (−)STobRV transcripts showed no biological activity.

We have now prepared synthetic plant gene constructions, designated SH+, ST+ and ST− according to the insert, using cloned STobRV complementary DNA segments (Fig. 1). SH+ contains an internal *Hae*III fragment from cloned STobRV cDNA (ref. 13) in an orientation which allows transcription to produce (+) strand RNA, whereas ST+ and ST− contain inserts which are trimers of the cloned permuted monomer cDNA of STobRV in orientations which allow transcription of (+) and (−)STobRV sequences, respectively. These inserts are fused to a constitutive promoter element from the cauliflower mosaic virus (CMV) 35S promoter and 3' terminal sequences from the nopaline synthase gene of *Agrobacterium tumefaciens* (Fig. 1). They were introduced into *Nicotiana tabacum* var. Samsun using *A. tumefaciens* T-DNA transformation and selection with a kanamycin resistance (*kan*^{r}) marker.

Transgenic plants were regenerated and tested for expression of STobRV transcripts by RNA blot analysis of leaf RNA. Plants producing detectable levels of transcript were chosen for further study. Autoradiographs prepared from RNA blots of transgenic seedling progeny of these regenerated plants (Fig. 2) show hybridization signals similar to those seen in tests of RNA from the original regenerants. The amounts of detectable STobRV transcripts in plants containing SH+ and ST+ StobRV gene constructions were higher than those containing the ST− construction. RNAs detected in the plant containing the SH+ gene construction were of ~1,500 bases, as expected for polyadenylated transcripts from this gene. The major transcript from the ST+ gene was STobRV RNA of monomeric length (359 bases), consistent with autolytic cleavage of the gene transcript. Minor bands in the autoradiograph are made up of products of partial autolytic cleavage. None of the regenerated plants containing the ST− construction produced RNA transcripts at comparable levels. A transformed plant which produced low levels of transcript was chosen for further study. The major transcript found in this plant was monomer length (359 bases), as expected from autolytic cleavage of concatameric (−)strand transcripts. Additional minor bands resulting from partial cleavage products and nonspecific binding to the excess of plant rRNA were also evident on the autoradiograph. These results provide the first proof that the autolytic cleavage of STobRV (+) and (−)strands seen *in vitro* can also occur *in vivo*. Further, in the transgenic plants there is no evidence of RNA-dependent RNA transcription in the absence of virus because only the transcript strands are detected using single-strand specific hybridization probes in RNA blots (Fig. 2).

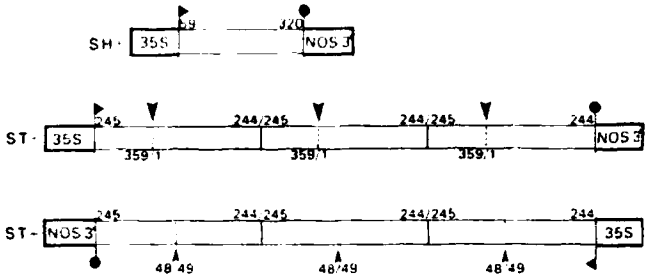

Fig. 1 Schematic representation of synthetic genes in transgenic tobacco plants (not to scale). For all genes, the CaMV 35S promoter and 3' terminal transcription termination and polyadenylation signals of the nopaline synthase gene[18] (NOS 3') were used for expression of STobRV sequences. Gene transcripts consist of RNAs containing a short leader sequence (4 bases) and the inserted STobRV sequence, followed by the 3' transcribed and polyadenylated region of the NOS gene (~1,200 bases). Nucleotides of the insert are numbered from 1 to 359 according to the sequence of the (+)strand of the satellite[7], and the gene constructions designated according to their cDNA insert as follows: SH+ (top) contains a *Hae*III fragment of STobRV cDNA, including nucleotides 57-319 of the sequence, and oriented to produce (+)strand transcripts. It lacks sequences required for autolytic cleavage *in vitro*. ST+ (middle) contains three direct repeats of a *Sau*3AI fragment of STobRV cDNA (refs 6 and 13), each repeat corresponding to a permuted full-length copy of STobRV, and oriented to produce (+)strand transcripts. The duplication of autolytic cleavage sites (between nucleotides 359 and 1) allows production of correctly terminated (+)strand STobRV RNA monomers. ST− (bottom) contains the trimer sequence described for ST+ oriented in the opposite direction. Duplication of (−)strand autolytic cleavage sites (between residues 48 and 49) allows production of (−)strand STobRV RNA monomers. Potential autolytic cleavage sites for both (+) and (−)strand RNAs are indicated by arrows. ►, ●, Respectively, 5' and 3' extents of the transcribed STobRV sequences.

Methods. The 35S expression vector p35SN was constructed by replacement of the NOS promoter of the expression vector pLGV2383 (ref. 18) with a *Bal*31 deleted CaMV 35S promoter extending from nucleotides −418 to −1 (ref. 19). Unique sites for *Bam*HI, *Sma*I and *Eco*RI are located downstream of the 35S transcription start and allow for insertion of sequences to be constitutively expressed in plant cells. Multimers of the *Sau*3AI insert from the cloned permuted monomer cDNA of STobRV (refs 6 and 13) were cloned into the *Bam*HI site of p35SN to produce permuted trimer constructions of both orientations. The *Hae*III fragment was also cloned into the *Bam*HI site using *Bam*HI linkers. Constructions were verified by restriction analysis and sequencing of the insert junctions. Each of the constructions was cointegrated between the T-DNA borders of the binary vector pGA470 (ref. 20) at unique *Hin*dIII sites and transferred to the *A. tumefaciens* strain LBA4404 (ref. 21) by triparental mating[22]. Constructions in *Agrobacterium* were verified by restriction and DNA blotting analysis. Leaf disks of *Nicotiana tabacum* var. Samsun line 5 (ref. 23) were infected with bacteria containing each of the constructions and transgenic plants recovered by selection on shoot regeneration medium containing 100 μg m^{-1} kanamycin[24]. Individual transformants with roots were analysed for expression of satellite RNA sequence transcripts by RNA blot analysis and then transferred to soil in a biosafety containment glasshouse. Plants were self-fertilized and R_1 seed collected for progeny analysis and virus infection testing of R_1 seedlings.

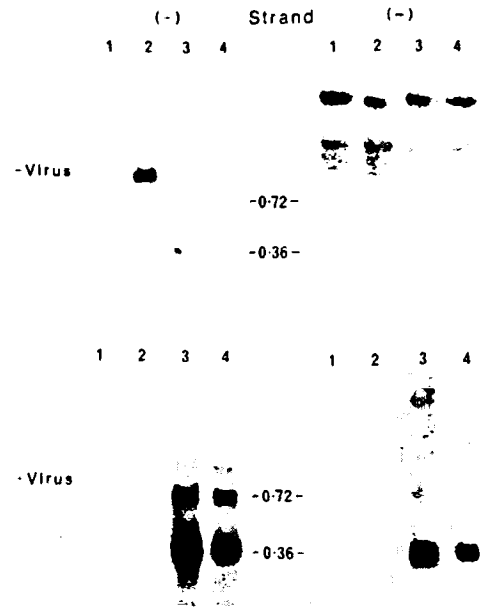

Fig. 2 RNA blot hybridizations showing STobRV RNAs in transgenic seedlings from regenerated plants. Samples are from infected leaves three weeks after inoculation with virus (+ Virus), compared with uninfected controls (− Virus). Single-strand probes were used to detect plus (+) and minus (−)strand sequences of STobRV. Results for representative plants of each genotype are shown. Lane 1, non-transgenic *N. tabacum* var. Samsun; lane 2, SH+ transgenic plant; lane 3, ST+ transgenic plant; lane 4, ST− transgenic plant. Mobilities of STobRV monomer (0.36 kilobases (kb)) and dimer (0.72 kb) size markers are shown.

Methods. RNA was extracted from leaf tissue (3 g) of representative plants. The leaf tissue was homogenized in a mixture of 4 ml buffer-saturated phenol, 8 ml chloroform, 4 ml 0.1 M NaCl, 0.1 M Tris Cl *p*H 9.5, 10 mM EDTA, 10% SDS, 140 mM 2-mercaptoethanol in a Sorvall Omnimix (30 s, maximum speed). We added 400 μl 3 M sodium acetate *p*H 5.5 and the sample was centrifuged to separate phases and pellet particulate matter. The aqueous phase was recovered and nucleic acids were precipitated with 3 vols ethanol. Pellets were resuspended in 0.8 ml H_2O and RNA samples (40 μg) were electrophoresed in 6% formaldehyde, 1.5% agarose gels[25]. Following two 30-min washes of the gel in distilled water, the nucleic acids were blotted overnight to Schleicher and Schuell BA45 nitocellulose using 20 × SSC transfer solution. After transfer the filter was baked under vacuum at 80 °C for 1.5 h. The hybridization method was that of Boyer[26] with modifications, using unlabelled single-strand M13 probes containing trimer (+) or (−) STobRV cDNA inserts for strand specific hybridization and radiolabelled M13 replicative form (RF) (10^8 c.p.m. μg^{-1}) as radioactive probe. X-ray film (Fuji RX) was exposed to the filters as follows: top exposures (− Virus): 1 day (plus strand) and 5 days (minus strand) exposure at −80 °C with intensifying screen; lower exposures (+ Virus): 10 min (plus strand) and 120 min (minus strand) at room temperature. The bands of high relative molecular mass seen for uninfected plant extracts hybridized with (−)strand probe are thought to result from non-specific trapping of probe on the filter by the vast excess of plant ribosomal RNA sequences. The bands comigrate precisely with those of the stained rRNAs and are also evident on long exposure of filters hybridized with (+)strand probe.

Seeds were collected from the regenerated plants: Stable incorporation of the transformed DNA was checked by 3 : 1 segregation of the linked kan^r phenotype in plants germinated from the seeds. To determine the effects of virus infection on the transgenic plants, twelve seedlings from each transformed genotype and twelve control (nontransgenic) seedlings were inoculated with TobRV virus four weeks after germination. Virus infection was established in all plants as judged by lesion development and by nucleic acid hybridization of leaf extracts using virus genome cDNA probes (data not shown).

STobRV (+) and (−) RNAs were assayed in virus-infected plants using single-strand cDNA hybridization probes (Fig. 2). After infection with TobRV, untransformed tobacco plants and the SH+ transgenic plants showed no alteration in levels of satellite RNA sequences, compared to uninfected plants. However, in plants containing the ST+ and ST− gene constructions there was amplification of satellite RNA sequences to high levels. This is consistent with replication of the STobRV sequence during the infection of the plants with virus.

A striking feature of the virus infections was the altered

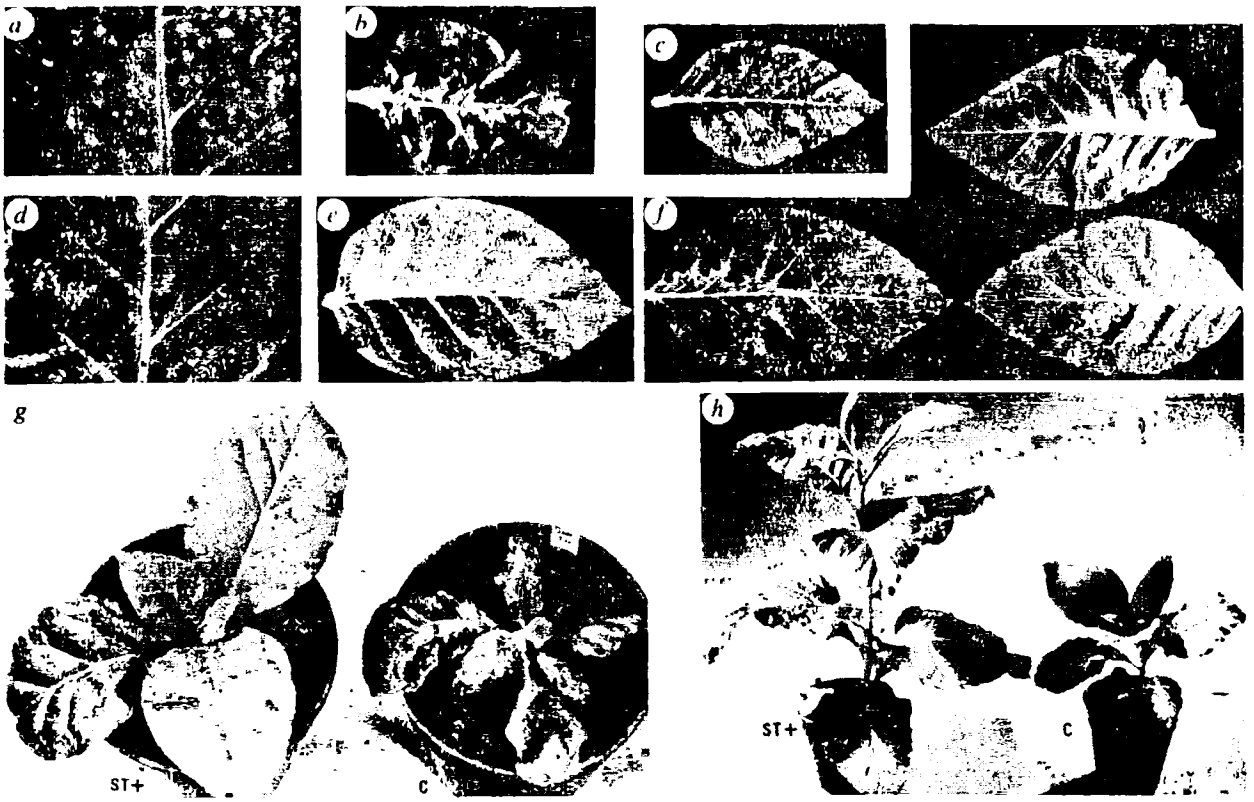

Fig. 3 Symptom development on *N. tabacum* var. Samsun non-transgenic control plants and transgenic plants containing an active ST+ gene construction at specific intervals after mechanical inoculation with TobRV. *a–c*, Leaf symptoms on non-transgenic control plants showing *a*, individual necrotic local lesions, 1 week after inoculation; *b*, strong systemic lesions and necrosis, 3 weeks after inoculation; *c*, mottled, small systemically infected leaves, 6 weeks after inoculation. *d–f*, Leaf symptoms on transgenic plants containing ST+: *d*, local lesions, 1 week after inoculation; *e*, symptomless leaf, 3 weeks after inoculation; *f*, symptoms, ranging from weak systemic reactions without necrosis to symptomless, from leaves 6 weeks after inoculation. *g–h*, Growth of non-transgenic plants (marked C) compared with that of ST+ transgenic plants ('ST+'), 3 weeks (*g*) and 6 weeks (*h*) after inoculation.

Methods. Seed progeny from regenerated transgenic plants and nontransgenic controls were germinated on damp vermiculite at 20 °C. After 3 weeks they were transferred to 13-cm pots containing loam and grown in a biosafety containment glass house (23 °C day, 15 °C night, natural daylength and lighting) for a further week. Inoculum was obtained from leaves of cucumber var. Spacemaster (Krempins Seeds, Armidale, Australia) mechanically inoculated with TobRV, and heavily infected as judged by severe stunting ~100 lesions per inoculated leaf. That the virus was free of satellite RNA was verified by nucleic acid hybridization of extracts from the cucumber plants. Fresh leaf samples (5 g) were finely ground in 10 ml of 0.1 M sodium phosphate buffer *p*H 7.0 at 4 °C, and used to infect the cotyledon leaves and expanded first true leaves of tobacco seedlings (100 μl per leaf) by mechanical inoculation using carborundum as abrasive. Following inoculation the leaves were rinsed with water and the seedlings grown under continuous light with 12 h 18–24 °C temperature cycles for four days. Seedlings were then returned to the biosafety containment glasshouse and monitored for symptom development.

symptom severity in transgenic plants which contained the ST+ and ST− gene constructions. Symptom severity was scored at 1, 3 and 6 weeks after inoculation of the seedlings with virus. Symptom development was similar for untransformed plants and those containing the SH+ gene construction. One week after inoculation, characteristic ringspot-shaped local lesions with severe necrotic centres were evident (Fig. 3*a*). Three weeks after inoculation, these plants showed severe systemic symptoms on all newly developed leaves (Fig. 3*b*) and stunting of the plants (Fig. 3*g*); after six weeks newly developed leaves were reduced in size and showed mottled symptoms characteristic of severe systemic TobRV infection (Fig. 3*c*). In all plants containing the ST+ gene construction, the primary lesions developed 1–2 days later than for nontransgenic plants and the centres of the lesions remained green, without necrosis (Fig. 3*d*); after three weeks new leaves seemed symptomless (Fig. 3*e*), with a mild systemic reaction only developing on some leaves after 5–6 weeks (Fig. 3*f*). At these later stages, leaf size was normal and plant growth was more vigorous than for the nontransgenic plants (Fig. 3*g* and *h*), and was similar to that of mock inoculated controls. The protection was maintained throughout the life of the ST+ plants, with flowering occurring when the plants were 10 weeks old.

In the ST+ transgenic plants the decreased symptoms are correlated with a lower level of replication of infectious virus compared with non-transgenic plants. When a hybridization probe prepared from cDNA of TobRV genomic RNA1 is used to assay the level of replicated virus genome in mechanically inoculated leaves the hybridization signal from ST+ plants is lower than that for non-transgenic controls (Fig. 4*a*). Furthermore, in the protected leaves higher on the ST+ plants there is no detectable virus, as judged by genome hybridizations, whereas it is present in the leaves of the systemically infected Samsun controls (Fig. 4*b,c*). The only detectable virus in the upper leaves of the ST− plants occurs in the few leaves which show weak systemic symptoms six weeks after inoculation (Fig. 3*f*). Even there it is only present in the basal regions of those leaves and is associated with amplification of satellite RNA.

Disease development in the plants containing the ST− gene construction was different, with the initial ringspot lesions (one week after inoculation) possessing necrotic centres similar to infected nontransgenic controls. However, later lesions and subsequent symptoms at three and six weeks were more like those of the ST+ plants and, as these plants progressed, they developed a resistant phenotype and their growth rate recovered. The amplification of both (+) and (−)STobRV RNA in the ST+ and

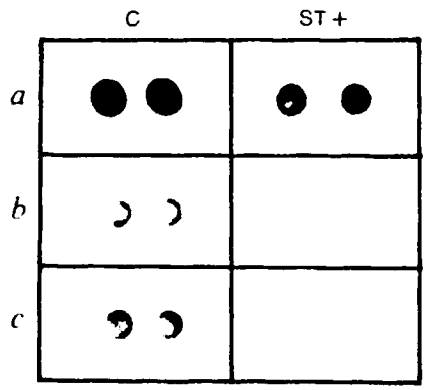

Fig. 4 Relative virus levels in leaves of an ST+ transgenic plant compared with a *N. tabacum* var. Samsun non-transgenic control plant four weeks after inoculation with TobRV. The autoradiographs show exposures of duplicate dots from equivalent leaf extracts following hybridization with radioactive probe complementary to TobRV virus genome. ST+, transgenic plant; C, non-transgenic plant. *a*, Leaf which was mechanically inoculated with virus, *b*, fourth leaf above inoculated leaf, *c*, eighth leaf above inoculated leaf.

Methods. Duplicate 20-mm-diameter disks were cut from leaves of ST+ transgenic and Samsun non-transgenic plants four weeks after infection by mechanical inoculation with TobRV as described in Fig. 3 legend. They were extracted by grinding in 1 ml 0.1 M sodium phosphate buffer *p*H 7.0, followed by shaking with 1 ml chloroform. After centrifugation to separate the phases, 3 μl of the aqueous extract was spotted in duplicate on to nitrocellulose membrane and baked at 80 °C for two hours. The filter was hybridized according to Waterhouse *et al.*[27] except that 10^7 c.p.m. of nick-translated pTB9 plasmid was used as probe. Plasmid pTB9 contains a 1.7-kb cDNA insert derived from TobRV RNA1 (from Mark Anderson, DSIR, New Zealand). X-ray film (Kodak AR) was exposed to the filter for 48 h at −80 °C with intensifying screen.

ST− (Fig. 2*b*) correlated with the resistant phenotype. Although this might have been expected for the ST+ transformed plants, the result was surprising for the ST− plants because *in vitro* transcripts of the complementary strand previously were not biologically active when inoculated onto plants with virus[13]. However, we cannot exclude the possibility of transcription of the ST− gene from an adjacent plant promoter, or of transcription of (−)strand RNA by host RNA-dependent RNA polymerases[14], to produce low levels of (+)strand STobRV sequences which were undetectable by RNA blot analysis of transgenic plants.

The production of novel virus resistance genes based on coat protein gene sequences of plant viruses has been shown for tobacco mosaic virus and alfalfa mosaic virus[15,16]. This paper demonstrates that sequences of a satellite RNA of tobacco ringspot virus can be used to construct genes which, when incorporated into plants, confer a resistance phenotype on the plant when it is challenged with tobacco ringspot virus. That this approach to the synthesis of resistance genes may be more generally applicable to other plant viruses is shown by the recent production of genetic resistance to a different plant virus, cucumber mosaic virus[17], using a satellite RNA unrelated to that of TobRV. A new approach to the synthesis of plant virus resistance genes is therefore now available.

We thank Drs John Randles and Richard Francki for the TobRV strain, Chris Howes for technical assistance; and Dr Paul Chu for advice on efficient mechanical inoculation of plants. J.H. was supported by CSIRO and Queen Elizabeth II postdoctoral fellowships. Experiments were carried out according to the guidelines of the Recombinant DNA Monitoring Committee, Department of Industry Technology and Commerce, Australia.

Received 17 June, accepted 12 August 1987.

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The role of clonal selection and somatic mutation in autoimmunity

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Polyclonal activation has been proposed as the reason that autoantibodies are produced during autoimmune disease[1-3]. This model denies a role for specific antigen selection of B cells and predicts instead a multiclonal population of unmutated or randomly mutated autoantibodies. We have found that the genetic features and clonal composition of spontaneously derived immunoglobulin G (IgG) antiself-IgG (rheumatoid factor (RF)) autoantibodies derived from the autoimmune MRL/*lpr* mouse strain are inconsistent with both the predictions of this model and the actual outcome of experimental polyclonal activation[4,5]. Instead we have found that MRL/*lpr* RFs are oligoclonal or even monoclonal in origin. They harbour numerous somatic mutations which are distributed in a way that suggests immunoglobulin-receptor-dependent selection of these mutations. In this sense, the MRL/*lpr* RFs resemble antibodies elicited by exogenous antigens after secondary immunization[6-8]. The parallels suggest that, like secondary immune responses, antigen stimulation is important in the generation of MRL/*lpr* RFs.

The MRL/*lpr* and other mouse strains which carry the autosomal recessive mutation *lpr* (ref. 9), are models of autoimmune disease[10-13]. The types of autoantibodies they produce bear a striking resemblance to those found in human systemic lupus erythematosus and rheumatoid arthritis patients[9,14-16]. One model to account for such autoantibodies suggests that self-specific antibodies are encoded by the germline *V* gene repertoire and are thus present in both normal and diseased individuals. In normal mice, self-reactive cells are suppressed, but in disease, an abnormal polyclonal B-cell activation occurs resulting in the pathological secretion of autoantibodies[1-3,17].