nonimmunized rabbit IgG ($P > 0.1$). The specificity of the PFU response was again demonstrated as no plaques were detected with either uncoated or ovalbumin-coated SRBC in either the direct or indirect assay. The reason for the detection of anti-HBs PFU in only one of six mice that received nonimmunized rabbit IgG for both injections is not known; however, this resulted in the standard error and the mean being equal. The numbers of direct and indirect PFU from the one mouse in this group were low and are probably not meaningful.

Although the standard deviation also exceeded the mean for direct PFU in groups treated with either antibodies to idiotype alone or with nonimmunized rabbit IgG and HBsAg, single-factor analysis of variance by ranks [Kruskal-Wallis test (*27*)] indicated that mean ranks of direct PFU differed significantly ($P < 0.05$) from the group receiving antibodies to idiotype and HBsAg. Such analysis was necessary because of the heterogeneity of the variances within the individual groups. The significance levels obtained by both the parametric two-tail Student's *t*-test and the nonparametric Kruskal-Wallis test were consistent.

We have shown that injection of antibodies to idiotype enhanced the anti-HBs response at the cellular level. An increased number of IgM anti-HBs–secreting cells was obtained by injecting antibodies to the idiotype before antigen exposure. The reason for the increase of cells secreting IgM, but not those secreting IgG, is not known. However, it may reflect the recruitment, by the antibodies to the idiotype, of accessory cells that aid in the induction of a primary anti-HBs response, thus increasing the number of IgM anti-HBs–secreting cells.

It was not surprising that anti-idiotype reagents would alter the anti-HBs activity in immunized animals, as similar results have been obtained in nonviral systems (*3, 8, 10, 12, 13*). Of potential importance was the finding that anti-HBs–secreting cells can be generated by administration of anti-idiotype reagents alone. Such induction of antigen-binding molecules in the absence of exposure to antigen does not usually occur in systems involving soluble antigen after treatment with a heterologous antibody to idiotype (*3, 10, 13*). Recently, however, antibodies to H-2 have been produced in mice by administration of a heterologous antibody to idiotype without alloantigen exposure (*2, 14*).

Anti-idiotype reagents provide an excellent means for modulating the immune response. Studies with these reagents should provide insight into the use of antibodies to idiotypes for manipulation of the immune response so as to provide enhanced protection of hosts from infectious agents. Such success has already been reported with anti-idiotype–induced protection in experimental trypanosomiasis (*11*).

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Nucleotide Sequence and Expression of the Diphtheria *tox*228 Gene in *Escherichia coli*

Abstract. *The complete nucleotide sequence of the diphtheria* tox*228 gene encoding the nontoxic serologically related protein CRM228 has been determined. A comparison of the predicted amino acid sequence with the available amino acid sequences from the wild-type toxin made it possible to deduce essentially the entire nucleotide sequence of the wild-type* tox *gene. The signal peptide of pro-diphtheria toxin and the putative* tox *promoter have been identified, a highly symmetrical nucleotide sequence downstream of the toxin gene has been detected; this region may be the corynebacteriophage* β *attachment site (*att*P). The cloned toxin gene was expressed at a low level in* Escherichia coli.

Diphtheria toxin is secreted as a single polypeptide by *Corynebacterium diphtheriae*, lysogenic for bacteriophage β^{tox+}, and is subsequently cleaved into two fragments, A and B (*1*). Fragment A blocks protein synthesis in most eukaryotic cells by specifically catalyzing an adenosine diphosphate (ADP)-ribosylation of elongation factor 2. Fragment B interacts with a membrane receptor for toxin and is essential for the delivery of fragment A into the cytoplasm. The toxin gene has previously been localized on the β-phage genome (*2, 3*), and the amino acid sequence of fragment A has been determined (*4*). Various mutant β phages have been isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) mutagenesis, and these encode nontoxic cross-reacting materials (CRM). Corynebacteriophage $\beta^{tox-228}$ carries the *tox*228 allele and encodes CRM228, which is serologically indistinguishable from the wild-type toxin (*5*). However, CRM228 is devoid of enzymatic activity and has a reduced receptor binding capacity.

We have cloned in *Escherichia coli* a 3.9-kb Bam HI fragment of DNA from the clear plaque-forming $\beta_c^{tox-228}$ in plasmid pBR322. We have localized the toxin gene on this fragment and have determined the complete nucleotide sequence of the *tox*228 allele and adjacent regions. The DNA sequence (Fig. 1) reveals a single open reading frame that is capable of encoding a protein with a molecular weight of about 61,000, corresponding to diphtheria toxin. The amino acid se-

quence predicted for the mature A fragment (residues 26 to 218) differs in only three positions from that of the wild type (Fig. 1). Two of the amino acid changes can be explained by single guanine (G), adenine (A) transitions resulting from the NG mutagenesis, the Gly → Asp exchange in position 104 (GGC → GAC) and the Glu → Lys exchange in position 187 (GAA → AAA) (*6*). Either one or both of these mutations are sufficient to completely abolish all ADP-ribosylating activity of fragment A. The third difference found in fragment A concerns the position of a Ser residue (Fig. 1) and may be due to allelic differences between the *C. diphtheriae* strains used [C7($\beta^{tox-228}$) in this work and PW8(ω^{tox+}) (*4*)].

A sequence of 342 amino acid residues is predicted for the diphtheria toxin B fragment. According to amino acid sequencing data for the wild-type fragment B from strain PW8(ω^{tox+}) (*7*) three amino acid changes can again be explained by point mutations due to NG treatment: Ser → Gly (position 222:AGC → GGC), Gly → Ser (position 456:GGT → AGT), and Asn → Asp (position 532:AAT → GAT) (Fig. 1). The latter two alterations may be responsible for the reduced capacity of CRM228 to bind to toxin receptors on cell membranes since the binding domain has been tentatively localized in the carboxyl terminal 17,000 daltons of the B fragment (*8*). Three additional amino acid changes (Fig. 1) cannot be explained by the NG treatment and, as above, could be due to allelic differences.

Diphtheria toxin is preceded by a leader peptide. The absence of any ATG (T, thymine) codon in the open reading frame upstream of fragment A, which ends at nucleotide 22 (Fig. 1), strongly suggests that translation initiates at the only GTG codon occurring in this region. The deduced amino acid sequence of the leader shows the typical pattern seen

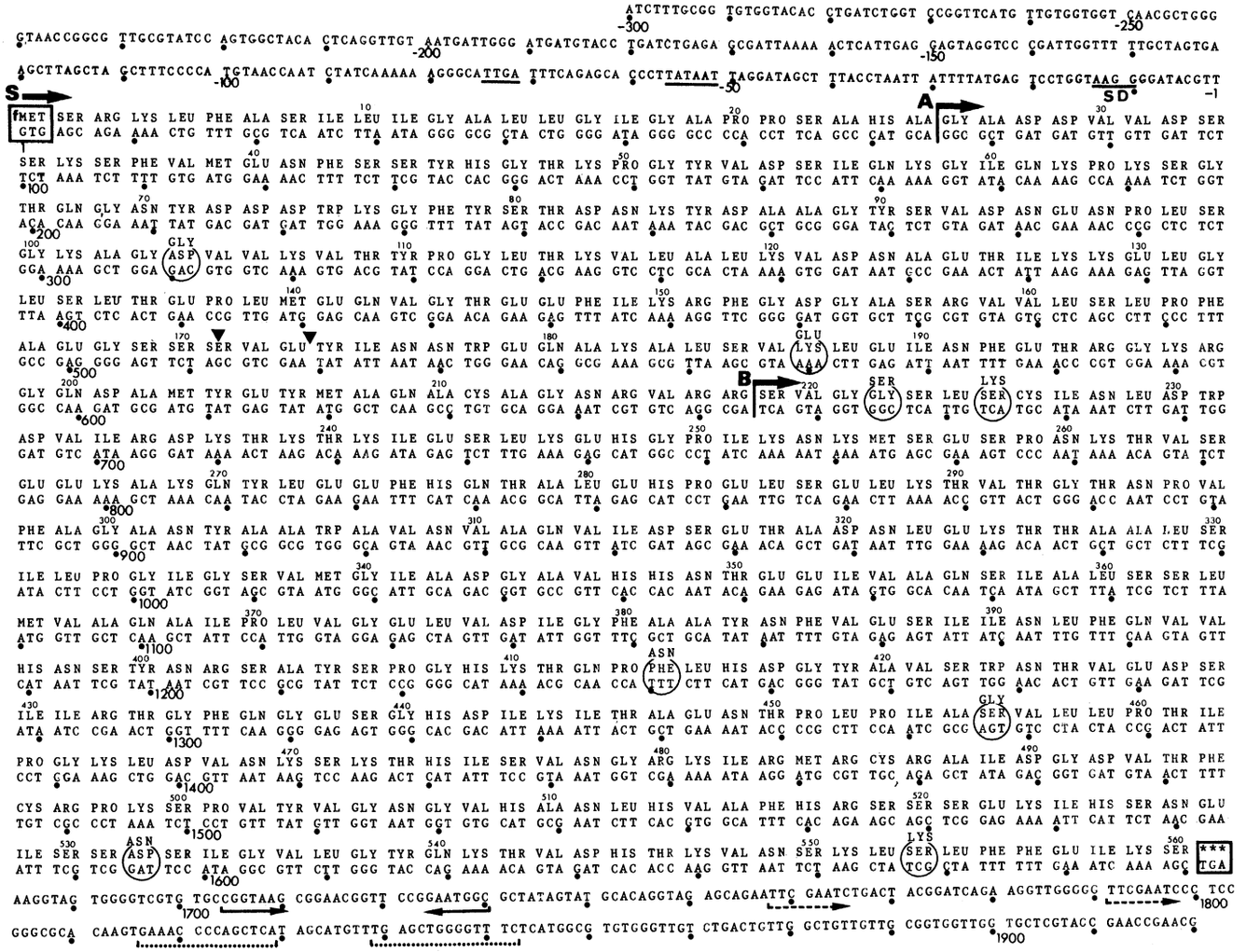

Fig. 1. Nucleotide sequence of the *tox*228 diphtheria toxin gene and of the flanking regions. Numbering starts with the first nucleotide of the translated sequence. In both coding and noncoding sequences dots mark every tenth nucleotide. The amino acid sequence deduced from the open reading frame is given above the nucleotide sequence with every tenth residue indicated. The beginning of the signal peptide (*S*) and of fragments A and B are indicated by arrows. The start and stop codons are in boxes. Amino acid residues which differ between CRM228 and wild-type toxin are in circles, and the corresponding wild-type residues are given. Ser-171 is missing in the A fragment sequence of reference (*4*) and instead placed between Glu-173 and Tyr-174 (▼). The consensus promoter sequences TTGA and TATAAT, as well as a possible Shine-Dalgarno sequence (*SD*) are underlined. Downstream of the gene, a 7-bp palindrome with mirror symmetry (——→), and 8-bp direct repeat (-----→), and a 14-bp palindrome with twofold symmetry (·····) are indicated. The nucleotide sequence was derived from clone pTD44 carrying the 3.9-kb Bam HI fragment from phage $\beta_c^{tox-228}$ DNA. More than 95 percent of the sequence was determined for both DNA strands (*21*). Promoter and leader region were also sequenced by the dideoxy method (*22*). They were verified by sequencing an independently isolated clone, pTD134, derived from a spontaneous clear plaque mutant of $\beta^{tox-228}$. Abbreviations for the amino acid residues are as follows: Ala, alanine; Arg, arginine; Asp, aspartic acid; Asn, asparagine; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; fMet, formylmethionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

with other bacterial signal sequences (*9*). A basic amino terminal region (Arg-3 Lys-4) is followed by a stretch of 19 "uncharged" amino acids, followed by His and Ala. The sequence at the cleavage site between the signal peptide and the mature fragment A, Ala − Gly, is again consistent with other sequences recognized by signal peptidases (*9*). The Pro residue found within six residues of the cleavage site also occurs very often in that position in other signal sequences (*9*). The detection of the leader is in agreement with the previous observation of Smith *et al.* (*10*), which was based on the in vitro completion of nascent polypeptide chains of membrane-bound polysomes extracted from *C. diphtheriae* in an *E. coli* translation system.

The *tox*228 coding region is preceded by sequences very similar to those found upstream of *E. coli* genes (*11*). These include a Shine-Dalgarno sequence (*12*), AAGG, 9 bp before the translational start, a TATAAT sequence in perfect agreement with the "−10" consensus sequence of *E. coli* promoters, and a TTGATT sequence related to the "−35" consensus sequence TTGACA (Fig. 1). Further characteristics of *E. coli* promoters also present in the putative diphtheria *tox* promoter are the A-rich sequence upstream of the "−35" region and the conserved A at the "−45" position. The major difference with respect to *E. coli* promoters is the short spacing of 12 bp between the "−10" and "−35" sequence, where 17 bp is the preferred distance in *E. coli*. The "−10" sequence forms part of a 9-bp inverted repeat (Fig. 2a). This region may possibly play a role in the iron-controlled regulation of diphtheria *tox* expression in *C. diphtheriae* (*13*), although this remains speculative.

The nucleotide sequence downstream of the coding region exhibits a large number of direct and inverted repeats, some of which are indicated in Figs. 1 and 2b. Transcription of the *tox* gene terminates in this region. According to previous restriction mapping (*2*), the 3′ end of the *tox* messenger RNA (mRNA) maps close to the Eco RI site located at position 1760. A 7-bp inverted repeat is found in this region (Fig. 2b).

Further downstream a perfect 14-bp inverted repeat is found and this is interrupted by 9 bp (Fig. 2b). We suggest that this unusual sequence is a component of the β-phage attachment site, *att*P, by which phage β integrates into the *C. diphtheriae* chromosome. The *att*P site has been mapped to within 50 bp of the Eco RI site at the end of the toxin gene (position 1760 in Fig. 2b) (*14*). Moreover, as a result of cloning and nucleotide sequencing of both the *C. diphtheriae* bacterial (*att*B) and the β-phage (*att*P) attachment sites, a common sequence containing the 14-bp inverted repeat and the 9-bp interruption has been detected (*15*). A similar structure is also found in the *E. coli* *att*B and the coliphage λ attachment sites where an 11-bp common core sequence is included in an inverted repeat (*16*).

The diphtheria *tox* gene can be expressed from its own promoter in *E. coli*. This was indirectly demonstrated with the use of two clones, *E. coli* pTD44 and pTD76, which carry the same 3.9-kb Bam HI fragment from $\beta_c^{tox\text{-}228}$ inserted in opposite orientations in the plasmid pBR322. A third clone, *E. coli* pTD134, which carries the 3.9-kb Bam HI fragment from a spontaneous clear plaque-forming mutant of $\beta^{tox\text{-}228}$, was used for comparison. In all three cases, CRM228 was detected in the bacterial lysates at 50 to 100 ng per milliliter of culture. In these experiments the *E. coli* strains were grown to A_{600} of 0.8, and the level of CRM228 expressed was determined by an enzyme-linked immunosorbent assay (ELISA) with horse antitoxin (data not shown). These results are in agreement with the observation that diphtheria toxin is expressed in an *E. coli* in vitro system programmed with β-phage DNA (*17*), and with those of Leong *et al.* (*18*) on the expression of diphtheria toxin fragment A in *E. coli*.

The level of expression of the diphtheria *tox* gene in *E. coli* is rather low. One possible explanation suggested by our data is that of weak transcription resulting from the short spacing of the "−10" and "−35" sequences in the *tox* promoter. Alternatively, translation may be slow because of the high incidence of rarely used codons in *E. coli*. For example, 36 of the 39 leucine codons in the *tox* gene correspond to rare codons. Two further examples are ATA (41 percent of all isoleucine codons in the *tox* gene, compared to 1 percent in *E. coli* genes so far examined), and the AGG codon for arginine, which occurs four times in the *tox* gene, but up until now has only been found twice in *E. coli* (*19*).

The complete nucleotide sequence of the wild-type diphtheria toxin gene can essentially be deduced from the *tox*228 sequence. Since there are only five point mutations leading to amino acid changes in the *tox*228 allele, the probability of silent mutants should not exceed the order of two to three for the entire gene. The probability of mutations in regulatory regions is also very low, since CRM228 is indistinguishable from the wild type in both its regulation and level of expression (*5*). It should now be possible, by site-directed mutagenesis and in vitro recombination, to generate specifically designed toxin molecules, a procedure that could lead to further under-

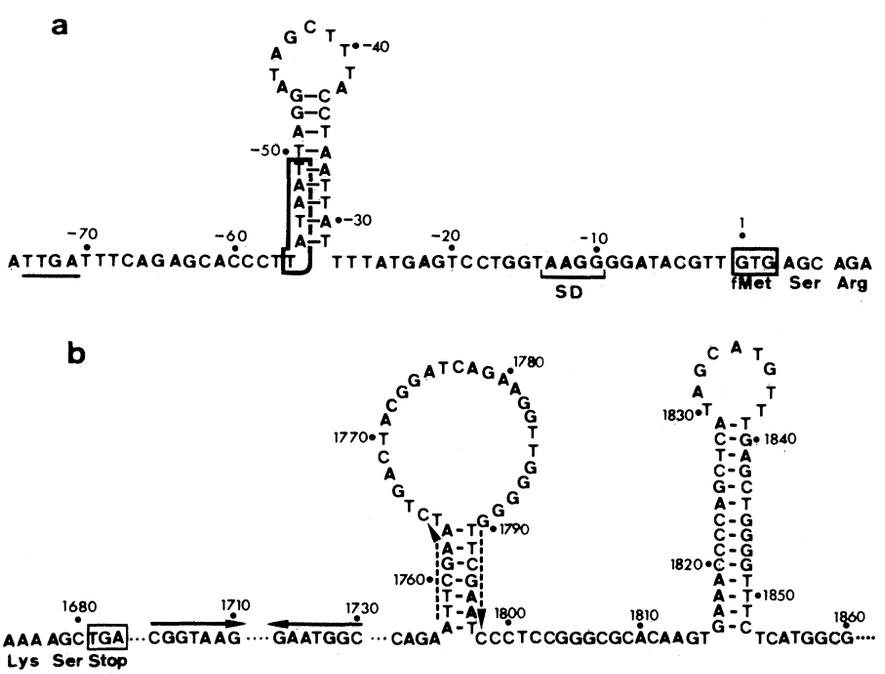

Fig. 2. Palindromic sequences in the flanking regions. The numbering of the nucleotide sequence is as in Fig. 1. (a) Promoter region. The "−35" and "−10" sequences, the Shine-Dalgarno sequence *(SD)*, and the beginning of the signal peptide are marked. (b) The 3′ nontranslated region. The end of the protein sequence is given and the palindromes given in Fig. 1 are indicated.

standing of the molecular action of diphtheria toxin.

Note added in proof: After communication of our results R. Delange (*20*) has reinvestigated the amino acid sequence of fragment A in the region corresponding to residues 171–174 (Fig. 1) where our sequences had differed in the position of a Ser residue. He now finds the sequence (Ser)-Val-Glu-Tyr in agreement with our data, that is, the originally published amino acid sequence (*4*) has to be corrected in this position.

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High-Level Expression in *Escherichia coli* of Enzymatically Active Harvey Murine Sarcoma Virus p21^{ras} Protein

Abstract. *The gene for the Harvey murine sarcoma virus (Ha-MuSV) p21*^{ras} *protein was fused to the amino-terminal portion of the bacteriophage* λ c*II gene on the expression vector pJL6. The fusion was such that transcription was controlled by the well-regulated phage* λ p_L *promoter, and translation initiated in the* c*II gene continued in frame into the* ras *gene sequences that code for p21. When the* p_L *promoter was derepressed, the* Escherichia coli *cells harboring the fusion plasmid synthesized 23,000-dalton protein, which represented more than 10 percent of the total cellular protein. This protein was chimeric and contained 14 residues, which were specified by the vector; these residues were followed by all of the amino acids that make up Ha-MuSV p21*^{ras} *except for four residues at the amino-terminal end. The protein appears similar to Ha-MuSV p21*^{ras} *in that it undergoes immunoprecipitation by monoclonal antibodies directed toward that protein, binds guanosine diphosphate, and is capable of autophosphorylation.*

The Harvey murine sarcoma virus (Ha-MuSV) is a replication-defective retrovirus capable of transforming cultured fibroblasts in vitro and inducing solid tumors in animals (*1*). It was isolated from solid tumors that developed in rats that had been injected with Moloney murine leukemia virus (Mo-MuLV). The transforming activity of Ha-MuSV is due to the presence of the v-*ras*^{H} oncogene (*2*), which codes for a 21,000-dalton (21K) protein called p21^{ras} (*3*). The *ras* gene of Ha-MuSV is closely related to sequences in the mouse and rat genomes that are called c-*ras* (*4*). It is likely that Ha-MuSV arose by recombination between rodent c-*ras* sequences and Mo-MuLV.

While the means by which p21^{ras} transforms cells remains unknown, it has several interesting properties. This protein binds guanine nucleotides (*5*) and is capable of autophosphorylation (*6*). Further study of the properties of this protein would be greatly facilitated by the availability of methods to prepare it in large quantities. One approach that has been applied to the protein products of several other viruses would be to produce the protein in bacteria. We chose to express the Ha-MuSV p21^{ras} by placing it in the plasmid pJL6 (*7*). This plasmid contains the 13 amino-terminal codons of the bacteriophage λ *c*II gene placed under the transcriptional control of the well-regulated phage λ p_L promoter.

pJL6 contains a single Hind III site adjacent to the Cla I site at the end of the *c*II gene on this plasmid. Translation beginning at the *c*II gene initiation codon should continue past this Hind III site further into the pBR322-derived sequences. This enzyme cleaves between the first and second base of a codon within the open reading frame defined by that translation start point. The sequence of the v-*ras*^{H} gene (*8*) predicts that Hind III also cuts that gene between the first and second base of a codon. Therefore, it was possible to create an in-frame fusion between the *c*II and *ras* genes by ligating a 880-bp Hind III fragment from plasmid H1 (*9*) containing most of the *ras* gene to Hind III–cleaved pJL6 DNA. A plasmid having the *c*II and *ras* genes in the same orientation was designated pJL*c*II*ras*1 and a plasmid in which the genes were in opposite orientations was called pJL*c*II*sar*1 (Fig. 1).

Plasmid pJL*c*II*ras*1 was introduced into *Escherichia coli* N4830 (*10*). This strain contains a highly deleted λ prophage carrying the mutant *c*I857 tem-

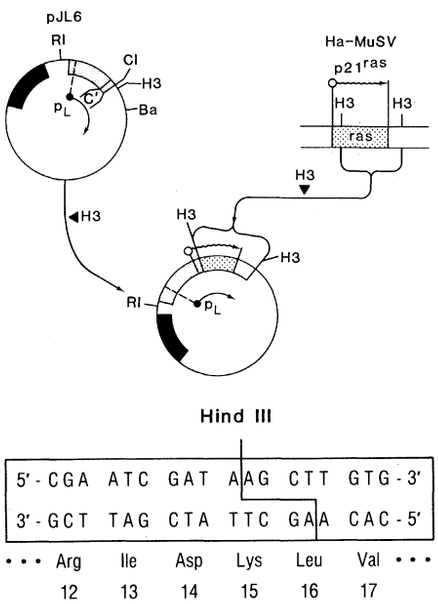

Fig. 1. Construction of pJL*c*II*ras*1. A 880-bp Hind III fragment [corresponding to positions 4040 to 4920, as shown by Ellis *et al.* (*2*)] containing most of the Ha-MuSV *ras* gene was isolated from plasmid H1 (*9*) and inserted into the Hind III site of pJL6 so as to generate pJL*c*II*ras*1. Symbols: solid segments, ampicillin resistance genes; heavily dotted segments, DNA derived from phage λ; and finely dotted segments, coding region for the Ha-MuSV p21^{ras} protein. The sequence shown is that of the *c*II-*ras* junction.