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J Genes for Heavy Chain Immunoglobulins of Mouse

Abstract. *A 15.8-kilobase pair fragment of BALB/c mouse liver DNA, cloned in the Charon 4A λ phage vector system, was shown to contain the μ heavy chain constant region ($C_H\mu$) gene for the mouse immunoglobulin M. In addition, this fragment of DNA contains at least two J genes, used to code for the carboxyl terminal portion of heavy chain variable regions. These genes are located in genomic DNA about eight kilobase pairs to the 5′ side of the $C_H\mu$ gene. The complete nucleotide sequence of a 1120-base pair stretch of DNA that includes the two J genes has been determined.*

An important feature of the immune system is its ability to generate, from a relatively small amount of genetic material, antibody molecules with many different antigen binding specificities. Antibody molecules contain heavy and light chains, each of which contains an amino terminal variable (V) region and a carboxyl terminal constant (C) region. The antigen binding site is formed by the V regions of both heavy and light chains (*1*). The C genes for the heavy chains and the two types of light chains, κ and λ, are all on separate chromosomes and have separate V region gene repertoires (*2*).

The mechanism for generating diversity of V regions for light chains in mouse has been thoroughly studied at the DNA level. These V regions are coded by two gene segments, V and J, which are joined by recombination at the DNA level to produce the completed V gene. The VJ and C genes are subsequently joined at the RNA level by splicing of the messenger RNA (mRNA) precursor (*3*).

The mechanism for generating diversity of V regions for heavy chains is not as well characterized at the DNA level as that for light chains. In mouse there are at least eight heavy chain constant region (C_H) types (μ, δ, γ_3, γ_1, γ_{2a}, γ_{2b}, α, and ε) (*4*), and all are encoded in the same chromosomal region (*2*). Analysis of protein sequences suggests that the V regions of heavy chains may be coded in separate V and J segments of DNA as the light chains are, and some protein V region sequences suggest that a third "D" segment may occur between V and J (*5*). In this report we present DNA sequence data establishing that heavy chain J regions are indeed encoded separately from the V and C regions. We have identified and sequenced two J genes and have shown that in germ-line DNA they are located about eight kilobase pairs (kbp) to the 5′ side of the $C_H\mu$ gene.

The general approach used in these studies was to isolate DNA clones for the heavy chain genes from shotgun collections made from mouse DNA ob-

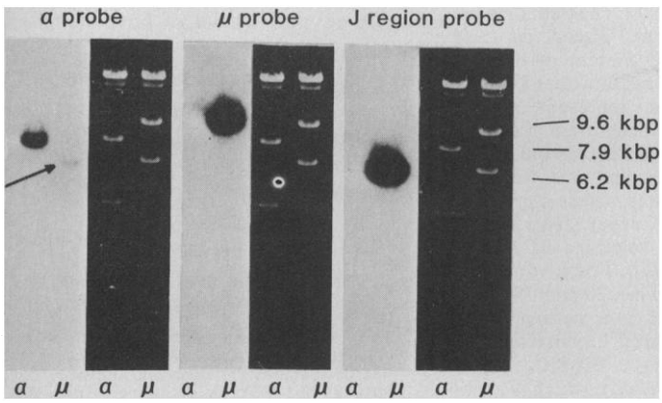

Fig. 1. Southern hybridization analysis of genomic $C_H\alpha$ and $C_H\mu$ genes. The two clones, Ch4A142.4 and Ch4A142.7, were digested with Eco RI and assayed by electrophoresis in triplicate on agarose gels in the channels designated α and μ, respectively. Photographs of the ethidium bromide-stained gels are shown in the right portion of each panel. In the left portion of each panel are autoradiographs of hybridization with three probes: (i) the VJα plasmid, pα(J558)[13]; (ii) the μ plasmid, pμ(3741)[9]; and (iii) the J region plasmid, pNN12. The arrow indicates the positive hybridization of Ch4A142.7 to the J segment seen with normal exposure with the VJα plasmid as probe. The other two panels are overexposed to show that there is no hybridization of either μ or J coding sequences to Ch4A142.4. By plaque hybridization experiments it was also shown that neither γ_{2b} nor γ_{2a} [which shares enough sequence homology to γ_{2b} that its presence would be shown with that γ_{2b} cDNA probe (*26*)] occur within 15 kbp of the 5′ side of the cloned $C_H\alpha$ gene (data not shown).

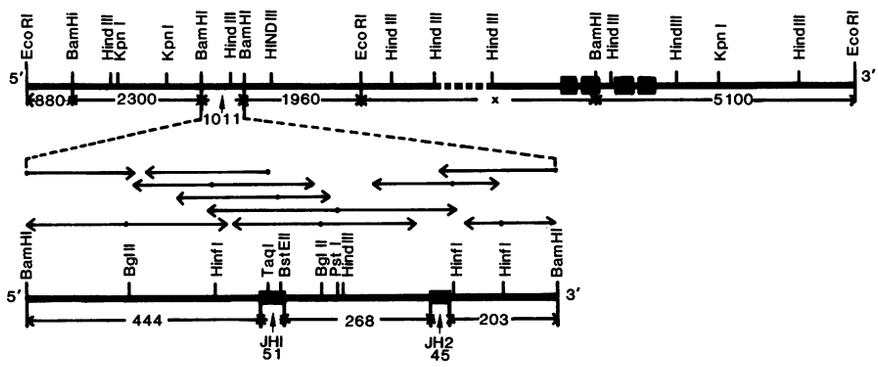

Fig. 2. Physical map of the genomic DNA insert in Ch4A142.7. The upper line shows the genomic DNA insert of Ch4A142.7 with the four domains of the $C_H\mu$ gene indicated as black boxes. Sizes of fragments are indicated below the line in base pairs. The size of the dashed Hind III fragment varies in different clones due to a tendency of this region of DNA to delete. The size of the interval marked × is 4450 bp in Ch4A142.7 but is about 7400 in genomic DNA. The lower line shows a more detailed map of the 1011-bp subfragment that was sequenced. The dots and arrows show the strategy used in sequencing. Fragments were end-labeled with ^{32}P at the dots, and the length and direction of sequence obtained is shown by the arrows. The restriction maps were obtained by electrophoretic analysis of DNA on agarose and acrylamide gels of single or multiple restriction endonuclease digestions. Southern hybridization analysis was used to identify fragments containing $C_H\mu$ or J_H sequences.

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tained from liver, a tissue uncommitted to the production of immunoglobulins. Clones of the μ and α C_H genes and J region genes were identified with the use of two plasmid probes. Both plasmids contained cloned complementary DNA (cDNA) copies of heavy chain mRNA's and were constructed with pMB9 as the vector (*6*). One plasmid [pμ(3741)[9]] was specific for the μ heavy chain C region gene and the other [pα(J558)[13]] was multispecific for a small portion of a heavy chain V region (V_H), for a complete J region, and for the α C_H region.

Clones were isolated by the megaplate method from our previously described shotgun collection of partially Eco RI-digested BALB/c liver DNA fragments in the phage Charon 4Aλ cloning vector (*7, 8*). Clone Ch4A142.7 contains two genomic Eco RI fragments, one consisting of 9.6 kbp and the other of 6.2 kbp. This clone showed positive hybridization by Southern analysis (*9*) to both the μ and the VJα probes (Fig. 1, A and B). The $C_H\mu$ probe hybridized to the 9.6-kbp fragment of Ch4A142.7 but not to the 6.2-kbp fragment. A portion of the VJα probe hybridized to the 6.2-kbp fragment of Ch4A142.7 (arrow, Fig. 1), which we subsequently showed to contain J region genes (see below).

In Ch4A142.7 and four other isolated clones, the sizes of the μ-hybridizing Eco RI fragments ranged from 9.3 to 10.4 kbp (*10*) in contrast to the result of genomic Southern hybridization analysis on our BALB/c liver DNA, which showed the μ gene to be on a single 12.5-kbp fragment. This suggests that during cloning, deletions ranging in size from 2.1 to 3.2 kbp have occurred. The site of these deletions mapped to the 5′ side of the $C_H\mu$ gene within the dashed Hind III fragment shown in Fig. 2 (*11*). The ready occurrence of these deletions indicates the probable presence of a region of repetitive DNA.

To confirm the presence of the μ gene in Ch4A142.7 and to establish its location and orientation, we constructed het-

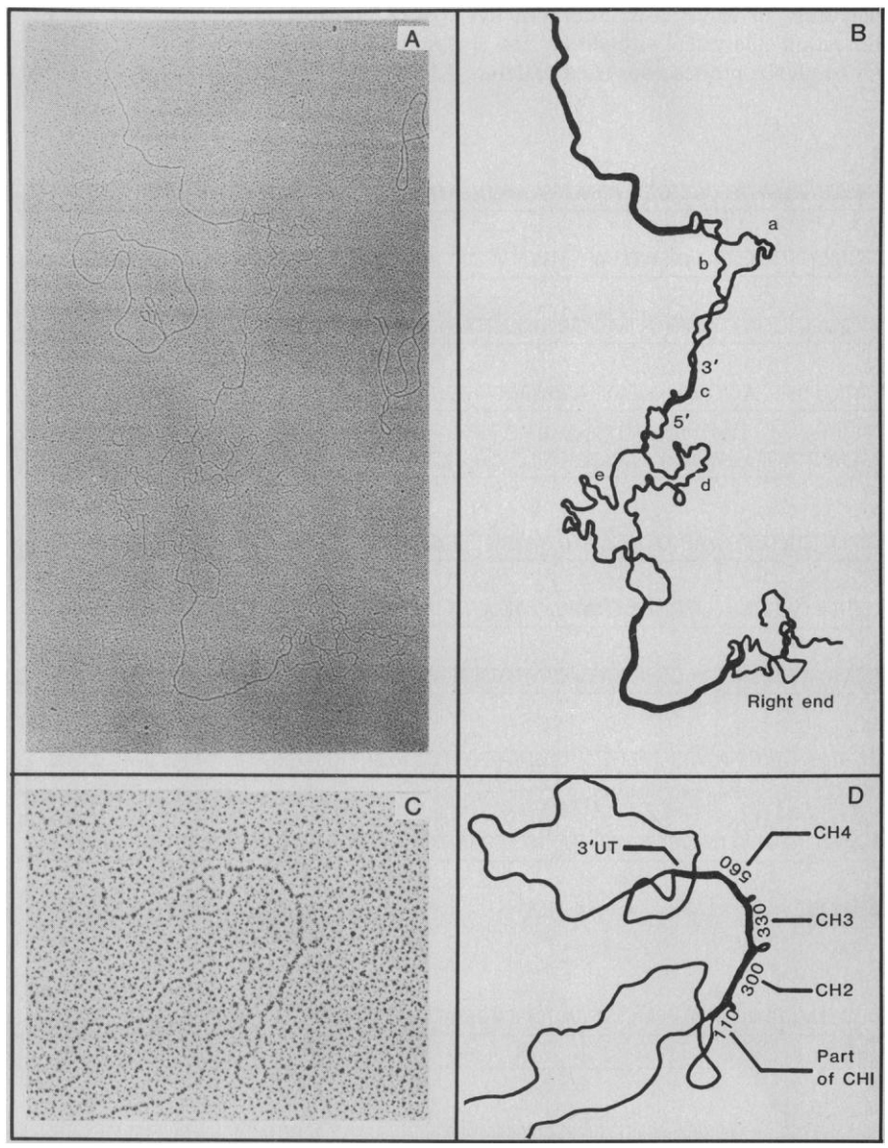

Fig. 3. Heteroduplex between Ch4A142.7 and Ch10pμ-u. (A) Electron micrograph. The heteroduplex tester phages were constructed by linearizing the μ cDNA plasmid by cutting it at the Hind III site of pMB9 and inserting it into phage vector Ch10 (*13*). Both the phage and plasmid have only one Hind III site, and no Hind III site is present in the μ cDNA. Thus, only two types of recombinants were produced, with the μ cDNA plus pMB9 cloned in each of the two possible orientations. According to our convention, the heteroduplex tester that contains the 5′ end of the μ gene closer to the left arm is termed Ch10pμ-n, and the one with the μ gene in the other orientation is called Ch10pμ-u. The left arm of Ch10 is identical to the left arm of Ch4A, but the right arms of the phages differ in known places. Consequently, when a clone in Ch4A is hybridized with a Ch10 tester, heteroduplexes are easily analyzed with the electron microscope. Thus, the orientation of the cloned fragment in Ch4A can be determined by establishing which of the two tester phages produces an internal region of homology, and the position of the homologous region within the fragment of cloned genomic DNA can be determined by measuring the single-stranded segments. Since the orientation of the genomic insert must be the same as the tester, we conclude that the 3′ end of the $C_H\mu$ gene in Ch4A142.7 is closer to the left arm of Ch4A. Similarly, it was shown that the $C_H\alpha$ gene in Ch4A142.4 is in the u orientation (data not shown). (B) Interpretative drawing. Lengths were measured on 15 molecules and are given in kilobase pairs ± standard error; the colicinogenic plasmid Col El was included as a length standard. (a) (5.6 ± 0.1 kbp) and (e) (10.6 ± 0.2 kbp) are the nonhomologous portions of Ch10pμ-u. (b) (3.5 ± 0.07 kbp) and (d) (9.5 ± 0.2 kbp) are the 3′ and the 5′ flanking regions of the μ gene, respectively. (c) is the region of homology between the μ cDNA and the genomic $C_H\mu$ gene. (C) Enlargement of an internal region of homology that shows all three intervening-sequence loops. The two smaller loops were not always visible in every heteroduplex molecule. (D) Interpretive drawing. Lengths are shown in base pairs. The 3′ untranslated region is abbreviated 3′ UT. From 5′ to 3′ the approximate lengths of double-stranded DNA formed between the intervening sequences of the cloned μ gene are 110 ± 37, 330 ± 63, 300 ± 84, and 560 ± 42 bp. We consistently observed three small loops within the region of homology, all less than 500 bases, which probably correspond to three intervening sequences. The simplest interpretation of these observations is that these segments contain the cloned portions of C_H1, C_H2, C_H3, and C_H4 plus the 3′ untranslated region, respectively. (Since only part of C_H1 is present in the μ cDNA, the hybridizing region of C_H1 is shorter than would be expected for a complete domain.) The μ gene is located 3.5 kbp from the Eco RI cloning site at the left end of Ch4A. The length of genomic DNA that hybridizes to the μ cDNA is about 1300 bases, suggesting that the entire C region is present in the cloned genomic piece. This information is in agreement with observations by others (*27*).

eroduplex tester phages containing the linearized μ cDNA plasmid inserted into phage vector Charon 10 (Ch10) (*12, 13*). The analysis of the heteroduplex between Ch4A142.7 and a heteroduplex tester phage of known orientation showed that the μ gene is present in Ch4A142.7, with its 3′ end closest to the left arm of Ch4A. We consistently observed four domains (in the form of double-stranded DNA) and three intervening sequences (in the form of small loops within the region of homology) (Fig. 3).

A restriction endonuclease map of Ch4A142.7 DNA (Fig. 2) was determined by analysis of single and multiple digests with a variety of restriction endonucleases. In each case, Southern hybridization analysis, with either the μ or VJα cDNA probes, was used to determine which fragments contained coding sequences. The sequences of Ch4A142.7 which hybridized to the VJα probe were confined to a 1-kbp Bam HI restriction fragment located 5.3 kbp to the 5′ side of the μ gene. This 1-kbp Bam HI restriction fragment was recloned into pBR322 (*14*) to facilitate the DNA sequencing of this region. This plasmid is called pNN12 (*15*).

We established that pNN12 (and, therefore, the 1-kbp Bam HI fragment in Ch4A142.7) contains J regions but no known V region sequences by determining the entire nucleotide sequence of the fragment (Fig. 4A) with the method of Maxam and Gilbert (*16*). Within the Bam HI fragment we found one stretch (J_H1) of 51 nucleotides that matched the presumed J region of the VJα plasmid perfectly and a second stretch (J_H2) that, when translated into amino acids, corresponded to known amino acid sequences of other presumed J regions of heavy chains (*5*). The second J segment differed in 11 of 45 nucleotide positions from the first. The DNA sequences adjacent to the two J regions in the Bam HI fragment were not homologous to either V region or C region sequences. These data consequently establish that at least two J regions are coded separately from the V_H and C_H genes and are located on the 5′ side of the $C_H\mu$ gene (*17*).

The experiment in Fig. 1, A and C, was designed to test whether the J_H cluster is repeated in front of other C_H genes. Since the J_H1 sequence matches the J558 α cDNA perfectly, we decided to test for its presence on the 5′ side of $C_H\alpha$. For this experiment we analyzed clone Ch4A142.4, a $C_H\alpha$ clone isolated from

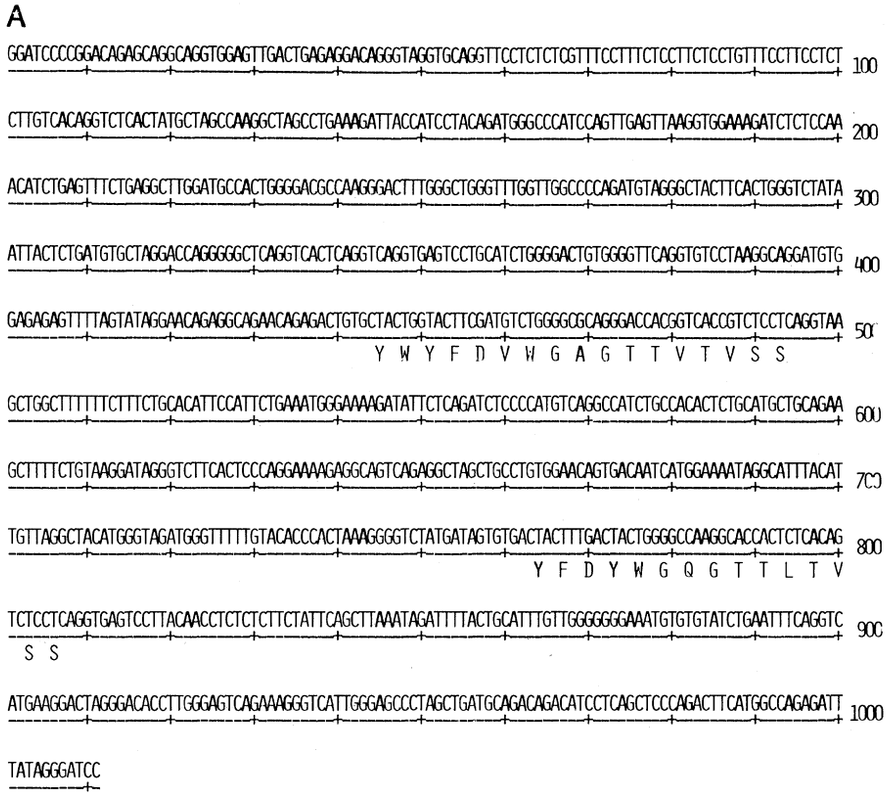

Fig. 4. (A) Complete nucleotide sequence of the 1011-bp Bam H1 fragment containing J_H1 and J_H2. The DNA sequence of the coding strand is shown on top with translated amino acids for the J genes below. Nucleotide numbering is indicated at the right of the line. The symbol + appears below every tenth nucleotide. (B) Sequence comparison of cDNA and genomic DNA segments from the BALB/c mouse. The nucleotide sequence of a portion of the variable region, including the J segment of J558 mRNA determined from the VJα plasmid, is presented on the first line with translated amino acids below. The amino acid numbering corresponds to the immunoglobulin M myeloma protein 104E which has the same J region amino acid sequence. Nucleotide and amino acid sequence of J_H1 and J_H2, including flanking DNA sequences, are taken from (A). The slashes on the 3′ side of the J genes indicate possible RNA splice sites. The conserved hepta- and decanucleotide sequences on the 5′ side of the J genes are indicated by bars. At the bottom of (B) are the J region protein sequences for Hdex 4, Hdex 5, and M315, which could be coded for by J_H2 (*5*). There are 17 known proteins of identical sequence that could be coded for by J_H1 (*25*).

the BALB/c shotgun collection with the VJα plasmid probe. Analysis of this clone with the α-specific heteroduplex tester showed the orientation of the insert (Fig. 3A) and that the genomic cloned segment contains C_H1, that part of C_H2 up to the Eco RI site in that domain, plus about 15 kbp of DNA on the 5′ side of $C_H\alpha$.

In this experiment, Eco RI–restricted Ch4A142.7 DNA and Ch4A142.4 DNA were analyzed by Southern hybridization. When the J region plasmid pNN12 was used as probe, hybridization was seen only to the 6.2-kbp fragment of Ch4A142.7. There was no hybridization with this probe to the $C_H\alpha$ clone. When the VJα cDNA plasmid was used as a probe, hybridization was seen not only to the 7.9-kbp fragment of the α clone but also to the 6.2-kbp fragment of the μ clone (arrow, Fig. 1). Thus we conclude that there are no J_H1 or J_H2 sequences within the 15 kbp on the 5′ side of the $C_H\alpha$ gene in our germ-line clone. The expression of immunoglobulin A must therefore require a rearrangement in which DNA from the 5′ side of $C_H\mu$ is translocated to the 5′ side of $C_H\alpha$.

To search for additional J regions, we sequenced 120 bases more of Ch4A142.7 DNA on the 3′ side of the Bam HI fragment. No additional J genes were found (data not shown) (*18*). Therefore, within a sequence of about 1120 bases, two J genes have been located, termed J_H1 and J_H2, which are separated by 268 base pairs (bp) and flanked on the left with 450 bp and on the right with about 320 bp of DNA without any other recognizable J_H sequences. Early and Hood (*19*) have hybridization evidence for a third J_H gene corresponding to MPC21 located within 1100 bp of the 3′ side of J_H2 (*18*), and Sakano and Tonegawa (*20*) have evidence for a third and a fourth J_H sequence also located on the 3′ side of J_H2 (*18*).

The sequences flanking the germ-line J_H genes are very similar to those surrounding light chain J regions (*21, 22*). On the 3′ side of all J genes [except for the nonfunctional J_κ (*21, 22*)] is an invariant GT (G, guanine; T, thymine) and a close match to the consensus sequence, AAGTA (A, adenine) found on the 5′ side of RNA splice sites (*23*). On the 5′ side of all J segments, including the J_H genes we have sequenced, there is a conserved palindromic heptanucleotide close to the canonical sequence CACTGTG (C, cytosine). Upstream from this sequence in all cases is a conserved decanucleotide related to PuGTTTTTGTA (Pu, a purine). For the J_κ and J_H segments, this separation is 11 bp (*22*). Thus, in all J genes studied so far, there appears to be a nearly integral number of helix turns between these conserved sequences.

The 5′ flanking sequences of the J_H genes are likely to be involved in the recombination event leading to V-J joining. Other investigators (*21, 22, 24*) have pointed out that on the 3′ side of germ-line light chain V genes there are sequences complementary to the conserved deca- and heptanucleotides that could lead to the formation of a stem-and-loop structure as a mechanism for bringing the V and J regions into close proximity in order to promote DNA recombination. Whether or not a similar mechanism could be proposed for heavy chain variable regions awaits the sequencing of a germ-line V_H gene.

Figure 4B shows nucleotide and amino acid sequence comparisons among J_H1 and J_H2 and various protein and cDNA sequences. The nucleotide sequence for J_H1 matches perfectly with 51 nucleotides of the V region from the VJα cDNA. It is likely the J_H1 gene was the same as that used to encode the α heavy chain protein produced by the J558 myeloma (*5*). The J_H1 gene also appears to have been used in 15 other myeloma proteins (*25*). By analysis of protein sequence data, the J_H2 gene could be used to code for the heavy chain proteins produced by three known myelomas, Hdex 4, Hdex 5, and M315 (*5*). Clearly the available protein sequences are not all accounted for by J_H1 and J_H2, but the number of additional J_H's required is difficult to estimate.

We have shown that at least two heavy chain J regions are coded by DNA sequences on the 5′ side of the $C_H\mu$ gene and that these DNA regions are not found within 15 kbp of the 5′ side of the $C_H\alpha$ gene. A logical extrapolation of the results we have obtained suggests that these two J regions are part of a single cluster of J genes used for all heavy chains. Our results provide the DNA sequences for two members of this cluster (*18*).

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8. Genomic DNA was partially digested with Eco RI and 200 μg of DNA (at a concentration of 1 mg/ml in 100 m*M* NaCl, 10 m*M* tris-HCl, *p*H 8, 5 m*M* EDTA) was applied to a gradient of 5 to 20 percent NaCl in 5 m*M* EDTA, 10 m*M* tris-HCl, *p*H 8. The gradients were centrifuged in a SW 41 rotor for 6.5 hours at 35,000 rev/min. Twenty-four fractions were collected and assayed by electrophoresis on 0.5 percent agarose gels. Fractions containing fragments in the 10- to 20-kbp size range were pooled. Ch4A DNA was treated with T4 DNA ligase to seal the cohesive ends and then cut with Eco RI. The NaCl gradients were then run in a similar fashion to separate the ligated arms of Ch4A from the two center inserts. Fractions containing only the arms were pooled. Pooled genomic fragments and purified vector arms were ligated together at a 1:1 molar ratio at 1 mg/ml and encapsidated by packaging in vitro (*10*) to make a shotgun collection of total genomic DNA.
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17. Our sequence for J_H1, J_H2, and the flanking sequences is in complete agreement over the 531 bases in which our sequence overlaps with that of Early *et al.* [P. Early, H. Huang, M. Davis, K. Calame, L. Hood, *Cell* **19**, 981 (1980)], except that we have an A at position 31 of our sequence which they do not find, and they find a T between bases 314 and 315 of our sequence, which we do not have.
18. After this report was submitted, we completed the sequence of 390 bases on the 3′ side of the 1-kbp Bam HI fragment in Ch4A142.7 so that the total DNA sequenced in this area is 1395 bases. In the area of new sequence we found J_H3, which begins 338 bases from the 3′ edge of J_H2. The new sequence, starting from the Bam HI site, is:

GGATCCTGGCCAGCATTGCCGCTAGGTCCCTCTCTTCTA
TGCTTTCTTTGTCCCTCACTGGCCTCCATCTGAGATAAT
CCTGGAGCCCTAGCCAAGGATCATTTATTGTCAGGGGTC
TAATCATTGTTGTCACAATGTGCCTGGTTTGCTTACTGG
GGCCAAGGGACTCTGGTCACTGTCTCTGCAGGTGAGTCC
TAACTTCTCCCATTCTAAATGCATGTTGGGGGGATTCTG
AGCCTTCAGGACCAAGATTCTCTGCAAACGGGAATCAAG
ATTCAACCCCTTTGTCCCAAAGTTGAGACATGGGTCTGG
GTCAGGGACTCTCTGCCTGCTGGTCTGTGGTGACATTAG
AACTGAAGTATGATGAAGGATCTGCCAGAACTGAAGCTT

The underlined bases code for J_H3, which is found in the A4, E109, and A47N protein sequences; J_H3 might also code for X44 and J539 protein sequences. Our sequence is in substantial agreement with the sequence of this area as determined by H. Sakano and S. Tonegawa (personal communication).
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25. J_H1 codes for M104E, Hdex 2, Hdex 3, Hdex 6, Hdex 7, Hdex 8, Hdex 9, Hdex 10, T15, S63, S107, Y5236, H8, M603, and M511. It is also possible that J_H1 might code for proteins W3207, M167, and T601, which each differ from J_H1 by one amino acid residue. Similarly, J_H2 might also code for X24, which differs from J_H2 by only one amino acid residue.
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28. We thank M. Fiandt for electron microscopy, A. Zrolka for technical help, O. Smithies for critical reading of the manuscript, and J. Schroeder for computer programming. We also thank P. Early, L. Hood, K. Marcu, H. Sakano, and S. Tonegawa for communicating results prior to publication. All experiments were done in accordance with NIH Guidelines on Recombinant DNA research. Supported by NIH grants GM 21812 (F.R.B.), GM06426 and AI16547 (P.W.T.), GM07131 (N.N.), and 5-T32-GM07133 (J.E.R.). This paper is No. 2430 from the Laboratory of Genetics, University of Wisconsin, Madison.
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Electrical Stimulation of the Midbrain Mediates Metastatic Tumor Growth

Abstract. *Pulmonary metastases were counted 10 days after female rats received tail-vein injections of Walker-256 carcinosarcoma cells. Previous observations that halothane anesthesia plus hind-limb amputation increases the number of metastases were confirmed. Amputation under the analgesia of electrical stimulation of the midbrain was found to increase metastatic activity. However, the stimulus-produced analgesia alone also increased the number of metastases. Systemically administered naloxone blocked the analgesic effect of midbrain stimulation but did not block the increase in the number of pulmonary metastases.*

Recent evidence suggests that certain anesthetic agents, used alone or followed by surgery, are immunosuppressive and associated with an increased number of pulmonary metastases in syngeneic mice inoculated with fibrosarcoma (*1*, *2*). Barbiturates alone increase the induction rate and the metastatic spread of several types of tumors in rodents (*3*). A recent report (*4*) documented accelerated growth of testicular cancer after cytoreductive surgery (there was no obvious explanation for this exacerbation of the disease). Halothane, however, does not increase tumor growth even with prolonged, repeated exposure (*5*). Our intent in this study was to isolate the role of surgery from that of inhalation anesthesia in the development of induced pulmonary metastases. Therefore, we amputated the hind limbs of rats anesthetized by electrical stimulation of the mesencephalic periaqueductal gray (PAG) region.

Stimulus-produced analgesia (SPA) has been elicited by delivering electric current to various brain locations, especially those around the mesencephalic cerebral aqueduct (*6*). Stimulation of the PAG region and nearby sites abolishes pain responses to noxious stimuli mediated by spinal cord reflex, yet does not affect motor activity. Morphine also blocks these reflexes (*7*). Both morphine analgesia and SPA can be impeded by the specific opiate antagonist naloxone (*8*). The analgesia elicited by stimulating the PAG region was more than sufficient for easy amputation of the hind limbs of our rats.

Although much of our data has been obtained from mouse studies, our neurosurgical experience in stereotaxic techniques is well developed with rats. We therefore chose the female Sprague-Dawley rat as the experimental animal. Before proceeding to the SPA experiments, then, it was necessary to recapitulate in the rat model the preliminary halothane and surgery studies done in the mouse.

Walker-256 carcinosarcoma cells, maintained in ascites form in Fischer rats, were harvested by sterile peritoneal tap and washed three times in phosphate-buffered saline (PBS). Cell viability, as determined by trypan blue dye exclusion, was greater than 95 percent in each experiment. Next, a dose-response curve for production of pulmonary metastases was established. On day 0, we injected 10^2 to 10^6 tumor cells in PBS (0.2 ml) into the tail veins of the rats after exposing them to ether for 30 seconds. Ten days later, the animals were killed with an overdose of ether, and the number of pulmonary metastases was counted by the India ink method of Wexler (*9*).

We chose a dose of 1×10^3 cells for further experiments because it produced a consistently low number of metastases. Anesthesia was induced with 4 percent halothane in oxygen for 1 minute and maintained with 1 percent halothane for 6 minutes (the length of time necessary to perform the amputations) (*10*). We found that the mean number of metastases in group 2 (tumor plus halothane) was similar to that in the controls (tumor alone). However, in group 3 (tumor plus halothane plus surgery), the number was markedly increased. The effect of surgery alone could not be deduced by following this protocol.

In order to evaluate the effects of surgery under SPA, we anesthetized the rats with ketamine HCl (150 to 250 mg/kg) and positioned them in a stereotaxic frame. A concentric 30-gauge electrode (*11*) was then implanted into the substantia grisea centralis at the junction of

Table 1. Number of pulmonary metastases in the different treatment groups (mean ± standard error).

Group	Treatment	Rats (No.)	Metastases	
			Raw data	Transformed data
1	Tumor	10	0.7 ± 0.2	0.64 ± 0.29
2	Tumor and halothane	10	1.9 ± 0.3	1.26 ± 0.25
3	Tumor, halothane, and surgery	9	17.1 ± 2.6	4.05 ± 0.70
4	Tumor and electrodes	7	1.1 ± 0.3	0.89 ± 0.34
5	Tumor and ESM (SPA)	9	15.9 ± 2.3	3.92 ± 0.63
6	Tumor, ESM, and surgery (SPA)	9	16.9 ± 1.8	4.05 ± 0.49
7	Tumor and naloxone	8	4.1 ± 1.0	1.91 ± 0.37
8	Tumor, naloxone, and ESM (no SPA)	8	12.6 ± 2.7	3.36 ± 1.21

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