Reports

Cloning of Herpes Simplex Type 1 DNA Fragments in a Bacteriophage Lambda Vector

Abstract. *DNA isolated from defective and nondefective virions of herpes simplex type 1 (HSV-1) (strain Patton) was digested with restriction endonucleases, and the resulting DNA fragments were inserted in the EK2 coliphage vector λgtWES·λB. The recombinant DNA was encapsidated in vitro under P4 maximum containment conditions. These λ-HSV1 hybrids were purified and amplified, and the DNA was isolated in the P4 facility. DNA, free of viable phage and bacteria, was removed from P4 conditions and analyzed. Represented among the hybrids studied to date are DNA fragments from about 50 percent of the normal HSV-1 genome. The hybrids derived from defective HSV-1 DNA fragments demonstrate the existence of many similar but not identical classes of defective genomes.*

Herpes viruses are common infectious agents of many vertebrates including man. Infection can persist in man in a latent form for extended periods with subsequent episodes of overt typical disease. A current topic of research and debate concerns the role of herpes simplex in tumor formation. The molecular biology of these viruses, particularly herpes simplex type 1 (HSV-1), is only now being established.

DNA extracted from HSV-1 virions is double-stranded, linear, and about 160 kilobase pairs (kbp) in size (*1*). The molecule can be divided in two unique portions, a large 133-kbp region (L) and a small 26-kbp region (S), each terminally redundant and capable of inverting with respect to the other by some, as yet unknown, mechanism (*2, 3*). Because of the L-S inversion, the HSV-1 genome exists in four approximately equimolar permutations in a given population of DNA molecules. Deletions and substitutions near the ends of the molecule have also been documented (*4*). In addition to these striking genome rearrangements, a more drastic type of sequence alteration readily occurs after repeated passage at high multiplicity. This is the appearance of defective HSV DNA molecules. One class of defectives studied to date has a much simpler genome that consists of tandem repeats of only one end (the S region) of the parental HSV DNA molecule (*5, 6*).

Our approach to analysis of this large DNA molecule and its fascinating genomic rearrangements has been to insert HSV-1 DNA fragments in a coliphage λ vector and to grow these hybrids in *Escherichia coli*. Only in this way can we pick out a single DNA fragment from a complicated mixture, effectively "freezing" it in one configuration so that it can be amplified in quantity and then studied in detail. In this report we describe the isolation of such λ-HSV1 hydrids and their preliminary characterization.

To insert HSV-1 DNA in a λ vector, we were required by the NIH Guidelines on Recombinant DNA (*7*) to use the most stringent biological and physical containment available: EK2-P4 combined biological and physical containment. We chose the certified EK2 vector λgtWES·B (λWESB) (*8, 9*) and used the P4 facility in Building 550, Frederick Cancer Research Center (FCRC), Frederick, Maryland. The experiments described here were submitted to and approved by the NIH Biohazard Committee (IMUA numbers 32 and 43).

The basic procedure for our experiments was as follows (*10*). The λ vector DNA was prepared and cleaved with Eco R1. The long arms of the vector DNA digested by Eco R1 were separated from the λB fragment by RPC5 chromatography so that plaque formation was dependent on the insertion of Eco R1 DNA fragments (Fig. 3, lane 3). The Eco R1-cleaved HSV-1 DNA was mixed with vector arms and joined by means of T4 DNA ligase (Miles or New England Biolabs). The ligated DNA was then brought into the P4 containment facility and packaged into phage particles by the in vitro method of Sternberg, Tiemeier, and Enquist (*11*). All subsequent procedures were done in the P4 class III cabinet.

Single plaques were picked and amplified in the disabled *E. coli* K12 host DP50*sup*F. The lysates were treated with chloroform, and the recombinant phage particles were concentrated with polyethylene glycol. The polyethylene glycol was removed, and the DNA was extracted from the phage particles by heating at 55°C for 2 hours in a mixture of 0.08*M* EDTA, 0.1 percent sodium dodecyl sulfate (SDS), and 200 μg of Proteinase K per milliliter. This treatment effectively inactivated the recombinant phages in that no viable phage or bacteria remained after sterility testing in the manner required by the Building 550 P4 Operations Manual. The crude DNA solutions were removed from the P4 facility according to the prescribed procedures. After treatment with ribonuclease and extraction with phenol the DNA was precipitated with ethanol, spooled on a glass rod, resuspended in buffer, and frozen for subsequent analysis.

The λWESB vector is particularly suited for insertion of Eco R1 fragments; however, fragments less than about 1 kbp or more than 15 kbp theoretically cannot be inserted in the vector because of size limitations of the phage head and the packaging mechanism. When nondefective HSV-1 DNA is cleaved by Eco R1, about 15 fragments are found (Fig. 1). Some are in submolar quantities expected from the four major permutations of the genome. Unfortunately, most of the fragments approach or exceed the theoretical 15-kbp size capacity of the vector.

We sought to improve our chances of inserting large Eco R1 HSV-1 fragments that were close to the theoretical size limit by in vitro packaging (*11, 12*). In this procedure, recombinant DNA is added to a mixture of partially assembled virions, and the added DNA is encapsidated in vitro to form viable phage particles. What is important is that larger DNA molecules are preferentially packaged over smaller ones. We expected that, given this size selection, we could pick up recombinant viruses with the largest DNA insertions possible for the λWESB vector.

Length Limit for Reports: The average length of individual Reports in *Science* has been steadily increasing. At the same time, the number of pages allotted to Reports has remained constant and cannot be increased. The net result has been that fewer Reports on fewer subjects are being published; many that receive excellent reviews are being rejected for lack of space. The overall rejection rate is more than 80 percent. In order to increase the acceptance rate for Reports in 1979 we plan to enforce the length requirements: one to seven double-spaced manuscript pages of text, including the references and notes, and two items of illustrative material (tables and figures) which together will occupy no more than half of a published page (30 square inches). After Reports are reviewed, those that are being considered for acceptance and that exceed the length limit will be sent back to the authors for shortening before a final decision is made. Reports that initially meet the length requirements will not be subject to this delay.

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In Fig. 2 we show a representative sample of the cloned Eco R1 fragments isolated from the HSV-1 genome. We digested the λ-HSV1 DNA samples with Eco R1 and fractionated them by agarose gel electrophoresis. The left and right arms of the vector can easily be discerned (Fig. 2, lane 2). In most cases, a single inserted Eco R1 fragment appeared. In several instances, two fragments were cloned at the same time (Fig. 2, lane 6). This probably reflected the size bias of the in vitro packaging system. The inserted fragment was initially identified by comigration with fragments of an Eco R1 digest of HSV-1 DNA; this assignment was verified subsequently by cleavage with several restriction enzymes, by hybridization of radioactive HSV-1 to the hybrids, and by hybridization of radioactive hybrid DNA to HSV DNA cleaved with several restriction enzymes (*13*). Because of the limited time available to us in the FCRC P4 facility (9 days), we were able to propagate only 14 Eco R1 hybrids derived from normal HSV-1 DNA for analysis. In these initial isolates, the L, M, and N fragments were repeatedly isolated as single and double fragments. We have succeeded in cloning two large Eco R1 fragments (Fig. 2, lanes 7 and 8), which we have identified as the D fragment (Fig. 2, lane 8) and the I fragment (Fig. 2, lane 7). The apparent size of the D fragment is about 17 kbp, yet the phage seems to be quite stable even though it is near the size capacity of the λ capsid. The I fragment (13.1 kbp) comigrates with the vector right arm fragment but can readily be identified by subsequent digestion with Bam HI or Kpn restriction enzymes (data not shown). Our inability to find hybrid phages carrying other large fragments or the small O fragment may not be significant because of the small numbers of hybrids analyzed.

One type of defective HSV genome is thought to be a tandem repetition of about 9 kbp from the S end of the normal HSV DNA molecule (*5, 6*) (see Fig. 1). Cleavage of defective DNA with Eco R1 yields a series of heterogeneous fragments superimposed over a background of the normal HSV Eco R1 fragments (Fig. 2, lane 9). Unlike normal HSV DNA, virtually all the defective Eco R1 fragments are within the size limitations of the λWESB vector. We sought, in this case, to insert in the vector a representative sampling of the various size fragments. We therefore fractionated the Eco R1 fragments derived from defective DNA by preparative agarose gel electrophoresis (*14*). Samples carrying DNA from the various size classes were ligated to the vector and packaged in vitro under P4 conditions. Typical cloned fragments are shown in Fig. 2, lanes 10 to 14, after digestion of the hybrid DNA with Eco R1 and subsequent agarose gel electrophoresis. More than 25 hybrids carrying Eco R1 fragments were studied, and in no case were the inserted fragments identical in size. We have characterized these fragments and, even though the size variation ranges from about 6 to 9 kbp, all the cloned fragments are derived from the extreme right end (the S region) of the normal HSV-1 molecule. The size differences appear to result from a variety of deletions and rearrangements. The Eco R1 fragments of defective HSV-1 DNA are heterogeneous in size but are closely related. Questions concerning how defective genomes arise and what role they play in the life cycle of herpes simplex type 1 await further study.

Because only 5 of 15 Eco R1 fragments of normal HSV-1 appeared in our initial collection of λ hybrids, we attempted to insert a wider range of frag-

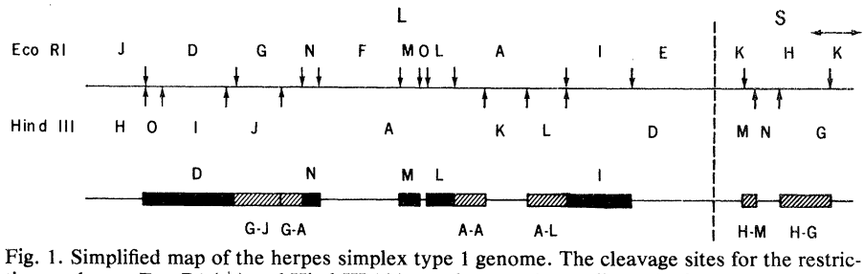

Fig. 1. Simplified map of the herpes simplex type 1 genome. The cleavage sites for the restriction nucleases Eco R1 (↓) and Hind III (↑) are given on the top line. The fragments produced by each enzyme are lettered according to the nomenclature of Skare and Summers (*3*). The large (*L*) and small (*S*) segments can invert with respect to each other around a joint region indicated by the vertical dashed line. Only one of four possible permutations is shown; several other fragments are generated from the three other permutations. The boxes on the lower line indicate the Eco R1 (—■■—) or Eco R1-Hind III (—▨▨—) fragments we have cloned in the λWESB vector. In naming the double cleavage fragments, the Eco R1 fragment gives the first letter and the Hind III site in that fragment gives the second letter. For example, in the S region, the Eco R1-Hind III fragment cloned was H-G. The double-headed arrow over the S region indicates that part of normal HSV-1 DNA found in the Eco R1 fragments of defective molecules we have cloned.

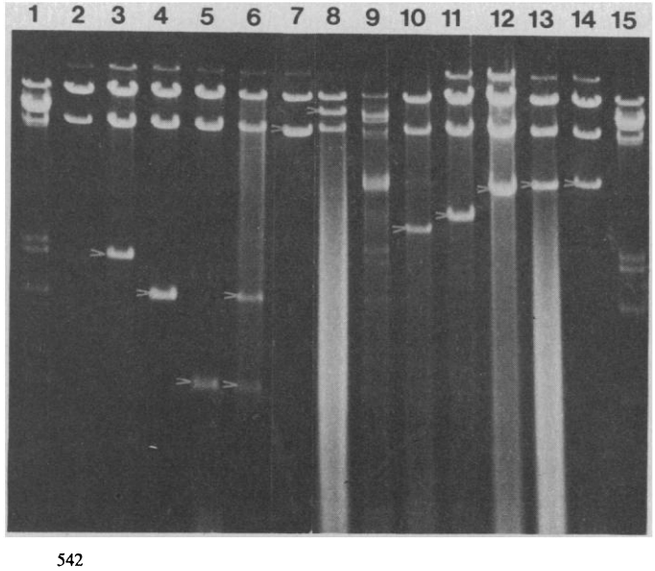

Fig. 2. Gel electrophoresis of Eco R1 restriction endonuclease products of λ-HSV1 recombinant DNA. DNA (1 to 2 μg/ml) was digested with 1 unit of Eco R1 enzyme (New England Biolabs), and was subjected to electrophoresis in a 1 percent agarose gel (*18*). Lanes 1 to 7 represent Eco RI restriction analysis of λ-HSV1 hybrids carrying Eco R1 fragments of the normal HSV-1 genome. The symbol (>) is adjacent to the cloned HSV-1 fragment. The faint band above the two λWESB arms in lanes 2 to 8 and 10 to 14 is a complex of left and right vector arms joined by annealing of the λ cohesive termini. (Lane 1) Wild-type HSV-1 DNA cleaved with Eco R1; (lane 2) λWESB purified Eco R1 arms; (lane 3) hybrid with HSV-1 Eco R1 fragment L; (lane 4) hybrid with HSV-1 Eco R1 fragment M; (lane 5) hybrid with HSV-1 Eco R1 fragment N; (lane 6) hybrid with two fragments, M and N; (lane 7) hybrid with HSV-1 Eco R1 fragment I (same size as right vector arm); (lane 8) hybrid with HSV-1 Eco R1 fragment D. Lanes 9 to 15 represent Eco R1 restriction analysis of λ-HSV1 hybrids carrying Eco R1 fragments of defective HSV-1 genomes. The defective DNA was isolated from virions after high multiplicity passage 13 (P13) and was purified as described (*6*). (Lane 9) P13 defective DNA cleaved with Eco R1; (lanes 10 to 14) representative λ hybrids with Eco R1 fragments from P13 defective DNA; (lane 15) wild-type HSV-1 DNA cleaved with Eco R1.

ments using a new approach with the λWESB vector and the restriction enzymes Eco R1 and Hind III. The cleavage sites of Eco R1 and Hind III in λWESB are shown in Fig. 3. The endonuclease Hind III cleaves many of the large HSV-1 Eco R1 fragments, and the resulting double digest (Hind III plus Eco R1) yields many more fragments of a size compatible with the λ vector. We designed the following procedure to insert fragments with one Hind III end and one Eco R1 end in λWESB (Fig. 3). No Hind III sites occurred in the left arm of the vector, but several sites occurred in the right arm. Cleavage of the complete λWESB vector (the central B fragment intact) with Hind III therefore contains, among many right arm fragments, a complete left arm fragment with a Hind III terminus. By adding this Hind III-cleaved vector to a tube containing the purified Eco R1 left and right arms, new fragment insertions other than simple Eco R1 fragments are possible. For example, if we added to this mixture fragments with one end derived by Eco R1 cleavage and the other end from Hind III cleavage, then a viable phage could be made by joining the fragment to the vector left arm derived by Hind III cleavage of complete λWESB and the Eco R1-cleaved right arm of the vector (see Fig. 3). Because several other combinations of ends are possible including some that cannot yield a viable phage, the specific infectivity of such mixtures is expected to be somewhat lower. In fact, we found that the specific infectivity was about tenfold lower than that found for purified Eco R1 vector arms and Eco R1 fragments. In Fig. 4, we show a typical analysis of a Eco R1-Hind III HSV fragment cloned by this method. The 8.8-kbp Eco R1-Hind III HSV fragment H-G (see Fig. 1) was partially purified by preparative gel electrophoresis and joined to the Eco R1-Hind III vector mixture described above. Ten plaques were picked and amplified. Analysis of the DNA indicated that three out of ten had indeed picked up a fragment of the proper size. The remaining seven phages had more complex patterns and have not been analyzed further. By hybridization of radioactive HSV-1 DNA to the hybrid DNA or by hybridization of radioactive hybrid DNA to HSV-1 restriction fragments, we could confirm that the cloned fragments were Eco R1-Hind III H-G. Using this method we have cloned and identified HSV hybrids carrying Eco R1-Hind III fragments A-L, A-A, H-M, D-J, G-J, and G-A.

The fragments cloned to date are shown in Fig. 1. More than 50 percent of the HSV-1 genome is now represented in λWESB hybrids. Of particular interest are (i) the hybrids with Eco R1 fragment N, which should carry a portion of the well-studied thymidine kinase gene (*15*); (ii) the hybrid carrying the Eco R1-Hind III H-G, which spans a portion of the S region that gives rise to defective genomes; and (iii) the whole series of hybrids which bear the defective HSV DNA fragments. The last-mentioned collection not only should provide insight to the mechanism of defective particle generation but the fragments may contain an origin of replication for the defective genomes. They also contain part of the

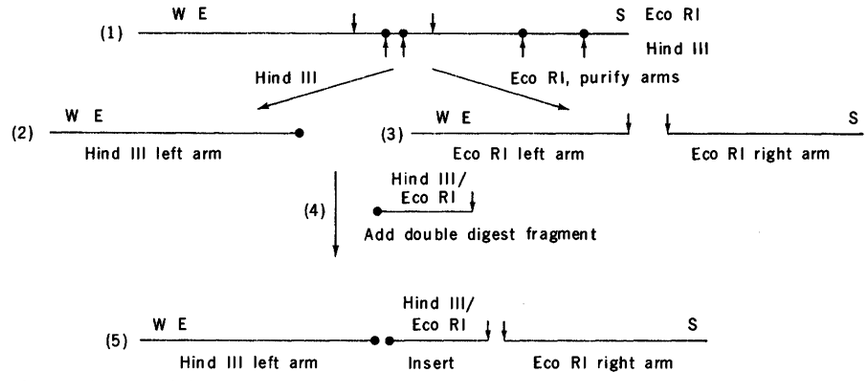

Fig. 3. The use of the λWESB vector for cloning fragments derived from double digests with Eco R1 and Hind III. The approximate cleavage sites for Eco R1 (↓) and Hind III (↑) in the λWESB vector are shown in lane 1. The left arm remaining after Hind III cleavage is shown in lane 2; the remaining fragments are not shown. The (•—) symbol at the end of a line indicates Hind III cohesive termini; the (↓—) symbols indicate Eco R1 cohesive ends. The λWESB vector cleaved with Eco R1 and the left and right arms purified away from the central B fragment is shown in lane 3. In lane 4 equal molar quantities (with respect to left arm fragments) of the mixtures depicted in lane 2 and lane 3 are combined with the Eco R1-Hind III fragment. Of a variety of combinations, the one shown in lane 5 should give rise to a plaque-forming hybrid phage.

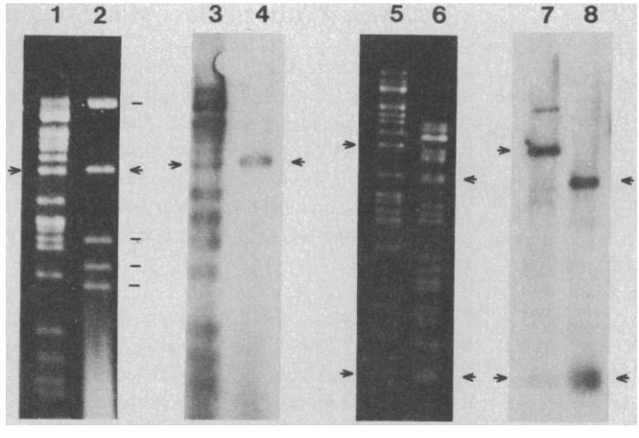

Fig. 4. Gel electrophoresis and hybridization analysis of an HSV Eco R1-Hind III fragment cloned in λWESB. The H-G fragment (Fig. 1) was partially purified from a Hind III-Eco R1 double digest of HSV DNA by preparative gel electrophoresis (*14*). The Hind III-Eco R1 vector was prepared in vitro as follows. A mixture of Eco R1 vector arms (*18*) and Hind III-cleaved λWESB (B fragment intact) was prepared each at 100 μg/ml in 20 m*M* tris, *p*H 7.4, 0.1 m*M* EDTA. The solution was heated at 70°C for 5 minutes and quickly cooled in ice immediately before use. The mixture (5 μl) was added to a tube containing the H-G fragment and the ligation buffer (*11*). T4 ligase (1 unit; New England Biolabs) was added and the mixture kept at 9°C for 20 hours, and then it was packaged into phage particles (*11*). Hybrids were purified as described (*10*). DNA (1 to 2 μg/ml) was digested with the enzyme indicated (*6*). (Lane 1) HSV-1 normal DNA digested with Hind III and Eco R1; the arrow indicates the H-G fragment. (Lane 2) Hybrid phage carrying the H-G fragment digested with Hind III and Eco R1. The arrow indicates the H-G fragment, and the dashes, the vector fragments. (Lanes 3 and 4) The same as lanes 1 and 2, except that this DNA was transferred to nitrocellulose paper (*13*) and hybridized with radioactive ^{32}P-labeled HSV-1 DNA. The arrows indicate the H-G fragment. The DNA was labeled by nick translation with DNA polymerase I (*19*). (Lane 5) Normal HSV-1 DNA cleaved with both Hind III and Eco R1. The arrows indicate the large H-G fragment and the small H-M fragment. (Lane 6) Normal HSV-1 DNA cleaved with Bam HI. The arrows indicate the large J fragment and the smaller X and Z fragments. (Lanes 7 and 8) The same as lanes 5 and 6 except that this DNA was transferred to nitrocellulose paper (*13*) and hybridized with nick-translated (*6*) ^{32}P-labeled λWESB hybrid phage DNA carrying the H-G fragment. The fragments expected to hybridize with the H-G fragment are indicated by arrows. The large fragment showing weak hybridization in lane 7 is a partial digestion product. Other faint bands in lanes 7 and 8 most likely represent partial digests, but as yet this has not been proved.

DNA sequence involved in the permutations of the L and S regions of the normal HSV genome. Because we can now make reasonable quantities of these specific HSV fragments quickly, economically, and without working with the infectious virus, structural analyses become much less tedious and complex. Furthermore, the separated DNA strands of cloned fragments for transcription analyses provide a tool not available for HSV before. The identification of proteins by the hybrid arrest method (*16*) with the use of in vitro translation of HSV-1 messenger RNA is simplified with these highly purified DNA fragments. Finally, we expect the new NIH Guidelines for Recombinant DNA Research (*17*) will lower the level of containment for these λ-HSV1 experiments so that these and similar hybrids will be available to most laboratories for further study.

Note added in proof: We have now cloned and identified two new Eco R1 fragments, H and G. In addition, we have cloned in the plasmid pBR322, the 3.4-kpb Bam HI fragment carrying the entire thymidine kinase gene.

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10. The λ-HSV1 hybrids were made by ligation of purified vector arms with Eco R1-cleaved HSV-1 DNA (*11*, *18*). The DNA mixtures were packaged in vitro (*11*) under P4 conditions. Initial plaques were formed on the *E. coli* K12 strain LE392 (*20*) at 38°C and then stabbed with toothpicks to another set of LE392 lawns for small-scale amplification at 38°C. The stabs were picked as agar plugs to Falcon 2063 tubes containing 1 ml of TMG (*11*) and two drops of chloroform. These primary stocks contained about 10^6 to 10^7 phage. For additional amplification, one drop of the primary stock was added to 0.2 ml of a twofold concentrated suspension of fresh overnight culture of DP50*sup*F (*8*) resuspended in 0.01*M* $MgSO_4$. After 5 minutes for phage adsorption, the mixture was added to 75 ml of LB broth containing thymidine (50 μg/ml) and diaminopimelic acid (100 μg/ml) required for growth of DP50*sup*F. The cultures were shaken vigorously at 38°C for 6 to 12 hours or until lysis occurred. They were then treated with 5 ml of chloroform at 38°C for at least 5 minutes and clarified by centrifugation at 4000 rev/min for 15 minutes. Phage particles were concentrated with polyethylene glycol 6000 (*11*, *18*). The phage precipitate was collected by centrifugation and resuspended in about 2 ml 0.02*M* tris-HCl, *p*H 7.4, 0.01*M* $MgSO_4$. The polyethylene glycol was removed with an equal volume of chloroform, and the mixture was centrifuged (4000 rev/min) for 15 minutes. The aqueous layer was removed and mixed in sterile serum vials with lysis buffer at a final concentration of 0.08*M* EDTA, 0.01*M* tris (*p*H 7.4), 0.1 percent SDS, and Proteinase K (0.2 mg/ml). The vials were tightly capped and heated at 55°C for 2 hours. A portion from each vial was tested in accordance with the Building 550 P4 Operations Manual [see (*7*)] for the presence of bacteria that could grow in LB broth supplemented with thymidine and diaminopimelic acid as well as for phages that could form plaques on a DP50*sup*F lawn. We estimated that about 10^{12} viable phage particles were in each vial prior to treatment. Only those vials with no bacteria or phage after 24 hours at 31°C in the sterility test were allowed to be removed from the P4 facility. Sterile vials were immersed in 0.1 percent Clorox solution for at least 5 minutes to decontaminate the vial prior to removal from P4. Once removed, the sealed vials were rinsed with mercaptoethanol buffer to inactivate residual Clorox and the contents treated with ribonuclease (50 μg/ml) (Worthington, pancreatic) for 60 minutes at 37°C, then sequentially extracted with one volume each of phenol, phenol : chloroform (1:1), and chloroform. Two volumes of ethanol were added to the aqueous phase and the precipitated DNA spooled onto a glass rod. The DNA (50 to 100 μg per each 75 ml of lysate) was dissolved in 20 m*M* tris-HCl, *p*H 7.5, 0.1 m*M* EDTA and frozen at − 20°C.
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20. LE392 is our stock number of strain ED8654, a gift from Dr. W. Brammar. The relevant genotype of this strain is *sup*E *sup*F *hsd*R$^-$M$^+$S$^+$ *met*$^-$ *trp*R *lac*$^-$.
21. We thank Dr. B. J. Graham for the defective DNA used; Dr. J. Seidman for advice and materials for some of the in vitro packaging preparations; Dr. David Tiemeier for advice and encouragement; Dr. Neil Wilke for the Bam HI physical map of HSV-1; and Dr. S. Nagle, T. Bryan, J. Brewer, and H. B. Stull for advice and technical support during our stay at the Frederick Cancer Research Center P4 facility.

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Radioimmunoassay of the Insulin Receptor: A New Probe of Receptor Structure and Function

Abstract. *A sensitive and specific radioimmunoassay for the insulin receptor has been developed employing receptor autoantibodies from the serum of a patient with insulin-resistant diabetes. The assay detects insulin binding sites at concentrations as low as 0.1 nanomolar; distinguishes between receptors originating from human placental membranes, human lymphoblastoid cells, and mouse liver membranes; and measures the receptor independently of its binding function. Down-regulation, or loss of binding after exposure to insulin, is associated with loss of immunoreactive receptor.*

Receptors for hormones, neurotransmitters, and other biologically active substances have been studied directly by measuring the specific binding of radiolabeled ligands (*1*). These studies have yielded information about the nature of ligand-receptor interactions and the role of receptors in physiology and disease. It is likely, however, that ligands interact with only a limited region of the receptor molecule, and techniques capable of probing other regions of receptors could help in elucidating receptor function. Toward this end, we have employed a highly specific antibody to the insulin receptor to establish a sensitive and specific radioimmunoassay for the insulin receptor molecule.

The receptor antibodies used in these experiments occur naturally in the serums of some insulin-resistant diabetics, who were described previously (*2*, *3*). The antibodies inhibit binding to both membrane-integrated and solubilized receptors (*2*-*5*), and specifically and completely immunoprecipitate solubilized insulin receptors (*5*). In addition, ^{125}I-labeled antibody binds to cells in proportion to their number of insulin receptors, and this binding is inhibited by insulin and insulin analogs in proportion to their affinity for the insulin receptor (*6*, *7*). Finally, the antibodies mimic a wide range of insulin effects on insulin-responsive cells in vitro (*8*, *9*). This evidence indicates that the antibodies bind directly to the receptor. It suggested to us that the antibody would be a suitable reagent for immunoassay of the receptor.

Insulin receptors were solubilized in Triton X-100 from human placental membranes as previously described (*10*).

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