Reports

Cloning Human Fetal γ Globin and Mouse α-Type Globin DNA: Preparation and Screening of Shotgun Collections

Abstract. *Shotgun collections of Charon 3A bacteriophages containing Eco RI fragments of human and mouse DNA were constructed with the use of in vitro packaging. Plaques were screened by hybridization, and globin-specific clones were isolated from both human (Charon 3AHs51.1) and mouse (Charon 3AMm30.5). The fragments cloned were detected in unfractionated genomic DNA by the Southern method of hybridization.*

The development of recombinant DNA techniques permitting clonal replication of eukaryotic DNA segments in bacteria has brought a revolutionary change in approach to genetic research. This set of techniques allows a DNA fragment containing a gene of interest to be replicated as a clone in a bacterium and opens the way to isolation of mammalian genes.

The first mammalian clones to be isolated in this way were complementary DNA (cDNA) copies of messenger RNA's (mRNA's) (*1*). Cloning of cDNA's takes advantage of the fact that comparatively pure mRNA's for specific genes can be isolated from cells specialized to produce large amounts of specific protein. However, this approach is limited to genes that produce an RNA product. While cDNA clones made from mRNA are useful, they lack intervening and regulatory sequences present in the genome.

A second major advance was the cloning of specific DNA segments partially purified from genomic DNA by column chromatography, R-loop centrifugation, or agarose gel electrophoresis (*2*). But physical DNA fractionation techniques are only capable of modest purification, and the effort required can be considerable.

The full power of the recombinant DNA technique lies in its application to unfractionated DNA. This approach (termed a "shotgun experiment") calls for the construction of a collection of recombinant DNA molecules which contains a large enough sample of cloned DNA fragments from a target genome to ensure that the desired single copy gene (or genes) is represented. In this approach, the entire purification of a DNA segment is accomplished by clonal replication. However, the method depends on the ability to recognize the DNA fragment of interest after it has been cloned.

The shotgun technique has been applied to organisms of low or moderate complexity such as *Escherichia coli* or yeast (*3*), as well as to the isolation of multiple copy genes from more complex organisms (*4*). In this and the following report (*5*), we describe the initial use of the shotgun strategy with mammalian genomes. We have used the procedure to isolate genomic clones for mouse α-type globin and human fetal γ globin DNA.

Both regulatory and technical difficulties have had to be overcome before the shotgun method could be used to isolate single copy mammalian genes. The U.S. regulations pertaining to the cloning of mammalian DNA have required that the cloning vectors and all techniques that might affect the biological containment of these vectors must be certified by the National Institutes of Health (NIH) (*6*). For these studies we have developed the cloning vectors Charon 3A, Charon 4A, Charon 16A, and Charon 21A (*7*) and techniques for in vitro encapsidation of the recombinant DNA molecules (*8*). These vectors and techniques have been certified by NIH as meeting the EK2 level of biological containment.

The second problem was the generation of very large collections of independent recombinant DNA molecules. Depending on the fragment size into which the genome is divided, from 10^6 to 10^7 individual clones are needed to overcome the statistical problem of sampling the mammalian genome. In principle, this could be done by scaling up each of the steps used in recombinant DNA experiments. However, a more desirable approach was to improve the efficiency of the techniques, particularly of the very inefficient transfection step in which phage DNA containing inserts of foreign DNA is introduced into *E. coli* cells. We therefore replaced this step with a modification of the technique for in vitro encapsidation of λ phage DNA (*9–11*) which led to an increase of about 100-fold in efficiency.

The strategy that was adopted for our initial experiments was to produce a shotgun collection by the simplest possible means. Our primary interest is the study of the human and mouse genomes, especially genes such as the globins, which show differential expression at different times of development and are involved in well-characterized genetic diseases (*12*).

Complete Eco RI digests of mouse and human DNA were the source of "random" genomic target molecules. These could be efficiently inserted into the cloning vector Charon 3A by ligation of their cohesive Eco RI termini. The use of complete Eco RI digests has the disadvantage that fragments exceeding the capacity of the vector will be difficult or impossible to clone. However, Charon 3A will accommodate a large range of Eco RI fragments [0 to 11 kilobase pairs (kbp) in theory]. Furthermore, some of the globin gene fragments produced by Eco RI digestion are known to be in this size range (*13*).

We expected that the most difficult step would be the screening of such a large population of phages to identify one carrying the gene of interest. Surprisingly, no new techniques were needed. We found that the radioactive plaque screening technique (*14*) could be scaled up by the use of cafeteria trays in place of petri dishes, so that a full mammalian genome could, in principle, be screened on two such plates.

Since probes for an increasing repertoire of genes are becoming available through cDNA cloning, one of the most attractive features of the plaque hybridization technique is the ability to screen for several genes at once. We initially decided to screen with a mixture of radioactive probes for four different genes. Although our efforts were frustrated by technical problems and we only obtained clones for globin genes in the first experiments, the use of multiple probes remains an important option for future work.

Target DNA's were purified by a method similar to that of Blin and Stafford (*15*). Mouse DNA was isolated from livers of random bred CD1 adults (Charles River Mouse Farms). In compliance with NIH regulations, human

0036-8075/78/1222-1279$01.50/0

Table 1. Comparison of three methods for introducing λ DNA into bacteria (*9–11, 18, 19*). The efficiency of maturation is defined as the number of plaque-forming units formed per input DNA molecule. (1 μg of λ DNA corresponds to 2×10^{10} molecules.) The efficiency of ligation is the ratio of plaque-forming units produced by digested and ligated vector and target DNA's to the number of plaque-forming units produced by undigested vector DNA. The fraction of output phages containing inserts varies, depending on the vector used. The amounts of target and vector Ch3A DNA needed to produce 10^7 clones were calculated on the basis of the efficiency of maturation. The volume of cultures needed to produce 10^7 clones was estimated on the assumption it would be possible to scale up the procedures directly.

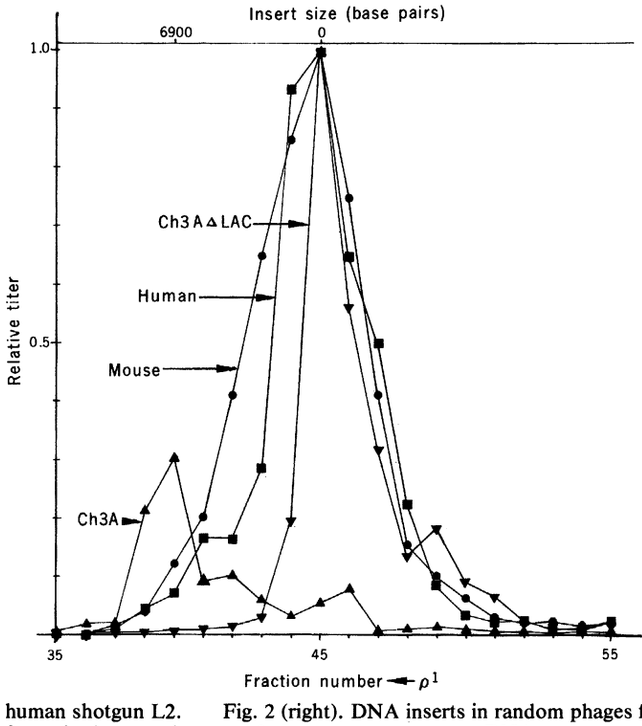
Method	Calcium shock	Spheroplasts	In vitro packaging
Efficiency of DNA maturation	10^{-6}	10^{-5}	10^{-3}
Efficiency of ligation	5×10^{-3}	5×10^{-3}	5×10^{-3}
Fraction of output phages containing foreign DNA	1/3	1/3	1/3
Number of λ DNA molecules needed to produce 10^7 clones	6×10^{15}	6×10^{14}	6×10^{12}
Mass of vector DNA needed to produce 10^7 clones	240 mg	24 mg	240 μg
Mass of target DNA needed to produce 10^7 clones	24 mg	2.4 mg	24 μg
Volume of cultures needed	400 liters	30 liters	2 liters

DNA was isolated from a primary cell culture of embryonic fibroblasts (*16*).

Before propagation of the Charon 3A vector, the Eco RI fragment carrying the *lac* Z gene, which had been inserted for safety testing, was removed by cutting with Eco RI, rejoining the cohesive ends, and transfecting spheroplasts. The resulting phage was designated Ch3AΔ1ac. Retention of all safety mutations was verified (data not shown). The target DNA and vector DNA with the λ cohesive ends annealed were digested separately with Eco RI and mixed in a 1:1 molar ratio of ends (vector: target, 7:1, by weight). The overall DNA concentration was 750 μg/ml. Ligation of DNA molecules at such a high concentration (*17*) favors the production of concatenated molecules whose lengths are many times that of λ. Most of the ligated DNA failed to enter a 0.7 percent agarose gel (data not shown).

The statistical distribution of fragments in such concatenates ought to follow a simple distribution with zero, one, and more than one target fragment occurring between vector fragments about equally often. In half these cases, vector fragments bracketing a target fragment would be expected to be oriented in the direction required to obtain infective particles. We would expect that clones containing two or more Eco RI fragments from unrelated portions of the target genome would not be uncommon, although clones containing large multiple inserts would be eliminated when the size of DNA exceeded the limit that could be fitted into a λ capsid.

Three techniques were considered for producing viable phage from naked recombinant DNA molecules: calcium shock transfection (*18*), spheroplast transformation (*19*), and in vitro packaging (*9–11*). Table 1 gives a comparison of the efficiencies of these techniques. Although the first two methods may be suitable for production of a few thousand phages, the volume of competent cells as well as the amount of vector and target DNA becomes prohibitive when a minimum of 10^6 or 10^7 individual phages must be produced from a ligation mixture. Therefore, we used in vitro packaging to introduce the recombinant molecules into bacteria. In this technique, cell-free extracts made from λ lysogens provide proheads, tails, and all other compo-



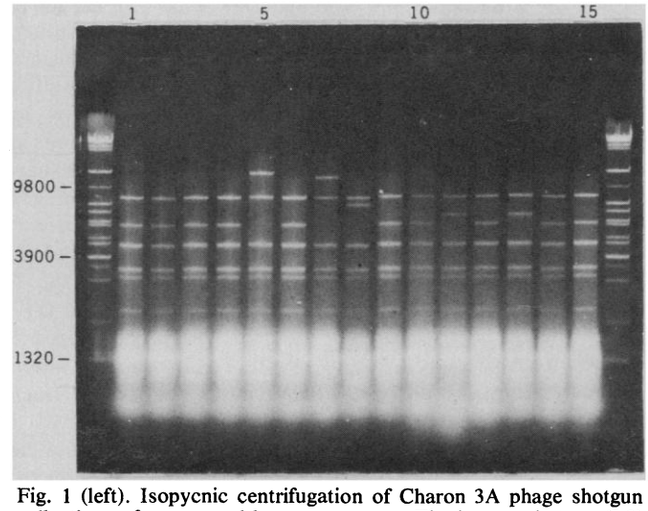


Fig. 1 (left). Isopycnic centrifugation of Charon 3A phage shotgun collections of mouse and human genomes. The human shotgun (L3) (■), the mouse shotgun (●), and a control tube containing Charon 3A (▲) and Charon 3AΔ1ac (▼) were centrifuged to equilibrium in a CsCl gradient of average density $\rho = 1.5$ g/ml in a Beckman SW 50.1 rotor at 30,000 rev/min. The tubes were punctured, and fractions were collected and titered. To distinguish Ch3A from Ch3AΔ1ac in the control tube, plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside were used (*7*). The peak fraction of each gradient was normalized to 1.0 before plotting. The results were virtually identical for the other human shotgun L2. Fig. 2 (right). DNA inserts in random phages from a human shotgun collection. Fifteen plaques were picked at random from the human shotgun L3. Lysates were grown, DNA was extracted from 2-ml portions (*29*) digested with Hpa I, and subjected to electrophoresis through 0.7 percent agarose gels that were stained with ethidium bromide and photographed under ultraviolet illumination. DNA fragments of known size were run in the outside channels. The sizes are 1320, 1990, 3110, 3600, 3900, 4590, 4925, 5690, 5950, 6760, 7560, 9570, 12400, and 19800 base pairs, a few of which are indicated in the figure. Samples 5, 7, 8, 11, and 13 show inserts manifested by changes in the migration of the band at 5.8 kbp. The sizes of the inserts measured from this gel ranged from 1 to 6.5 kbp. Comparable results were obtained for the other human shotgun L2 and the mouse shotgun.

nents needed to assemble phage in the test tube. Encapsidated DNA is then introduced into cells by infection.

For preparing a shotgun collection of phages containing random-length fragments, the packaging method must be efficient and its efficiency must be reasonably constant for molecules of different length. In addition, to preserve the safety features of the Charon vector system, there must be no packaging of endogenous λ DNA from the lysogens used to produce extracts, and no recombination between the vector DNA and this endogenous λ DNA.

Our technique (*20*) incorporates the basic procedure of Becker and Gold (*9*) which we modified by the use of extracts from strain NS428 described by Sternberg *et al.* (*11*) and by the addition of putrescine to the buffer (*25*) suggested by the work of Hohn and Murray (*10*). Use of the lysogenic strain NS428 to produce all extracts contributes to biological safety in two ways. (i) Since the cells and the prophage are recombination-deficient, recombination in the extracts between the Charon vector and the endogenous λ DNA is eliminated. (ii) Since the cells cannot produce A protein, prior packaging of endogenous λ DNA during induction of lysogens is blocked at the first step. There is no need to physically inactivate (by ultraviolet irradiation) prophage DNA from the extracts. Use of the spermidine-putrescine buffer along with isolated A protein allows a high efficiency of packaging for all size classes of recombinant molecules. Current NIH regulations require that these safety features should be verified for each set of encapsidation extracts (*21*).

Table 2. Construction of shotgun collections for mouse and human DNA. Charon 3AΔ1ac DNA and human or mouse target DNA were digested and ligated (*17*). The ligated DNA was encapsidated by in vitro packaging in the volumes shown (*20*). The titer of the packaged phages was then determined to ascertain the proportion of input phage DNA molecules encapsidated. The Ch3AΔ1ac control shows the efficiency of packaging achieved for DNA which had not been digested and ligated.

DNA	DNA (μg)		Total packaging volume (ml)	λ DNA equivalents in	Viable phage out	Packaging efficiency
	Ch3AΔ-lac	Genomic				
Human L2	20.4	2.52	1.57	4.97×10^{11}	4.55×10^{6}	9.1×10^{-6}
Human L3	61.2	7.56	4.71	1.49×10^{12}	3.76×10^{6}	2.5×10^{-6}
Mouse	40.8	5.04	3.14	9.88×10^{11}	2.1×10^{7}	2.1×10^{-5}
Ch3AΔlac control	0.7	0.0	0.208	1.7×10^{10}	7.2×10^{7}	4.2×10^{-3}

Three shotgun collections, two of human and one of mouse DNA fragments, were constructed (Table 2). In each case, more than 10^6 phages were produced. The efficiency of packaging of ligated DNA was between 10^{-2} and 10^{-3} of that obtained with untreated vector DNA.

Two methods were used to determine the distribution of inserts in these populations (Figs. 1 and 2). The first method, which can also be used preparatively to enrich for phages with inserts, was to subject samples of the shotguns to isopycnic centrifugation in CsCl gradients. Both mouse and human shotgun preparations exhibit broad density profiles with shoulders of density greater than that of the parent phage Ch3AΔlac (Fig. 1). From the area under the shoulder, we estimate that up to 40 percent of the phages in each shotgun contain inserts. However, very few appear to contain inserts larger than 6.7 kbp, the size of the inserts in the Ch3A marker (*7*).

The second method was to examine Hpa I digests of the DNA from plaques selected at random from the shotguns (Fig. 2). Inserts can be detected by changes in the mobility of the 5.8-kbp Hpa I fragment which carries the Eco RI cloning site. If the insert has Hpa I sites, more than one band will be produced. For example, Fig. 2 shows that 5 of the 15 candidates from human shotgun L3 had inserts and, in agreement with the density studies, the insert sizes ranged from 0.96 to 6.7 kbp. Thus the insert sizes observed covered a smaller range than the theoretical maximum capacity of Ch3A.

The Benton-Davis screening procedure consists of hybridization of radioactive nucleic acid probes to nitrocellulose replicas of lawns containing

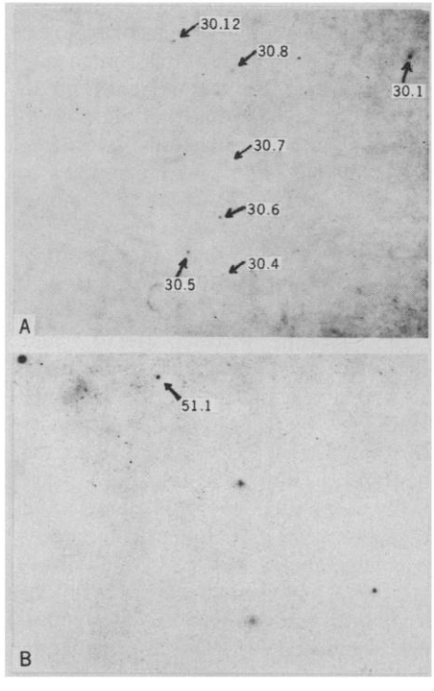


Fig. 3. Initial screening of mouse and human shotgun collections; 300,000 plaques each were screened by hybridization to nitrocellulose replicas (*22, 23*). (A) In the case of mouse, ^{32}P-labeled probes specific for rat insulin, rat growth hormone, and mouse α and β globin were used (*24*); poly(rA) was not included in the hybridization mixture. (B) For the human collection, a ^{32}P-labeled probe specific for human α, β, and γ globin was used; poly(rA) was included in the hybridization mixture (*23*). The figure presents approximately 25 percent of one of two autoradiograms taken from each filter. Arrows point to the signals that appeared on both films. Plaques were picked from beneath these signals and numbered as illustrated.

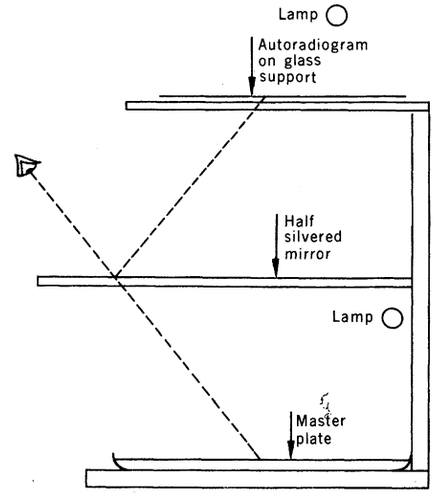


Fig. 4. The "Tucker" box. This device, based on a technique widely used for crystallographic model building, proved indispensable for picking candidates from megaplates requiring P3 containment. It allowed accurate picking of plaques under a laminar flow hood without contamination of the autoradiogram or the plate. Since frequent alignment marks were used (*22*), it also permitted compensation for the approximately 1 percent shrinkage of the nitrocellulose replica. A half-silvered mirror is supported midway between the top surface of the megaplate and the autoradiogram, which rests on a sheet of glass. The sides of the box, which provide support, are omitted from the drawing. The box was designed to fit easily within a laminar flow hood. Variable transformers were used on the lamps to allow light intensity adjustment for best illumination. The observer sees images of the autoradiographic spots and alignment marks in the same plane as the top agar of the megaplate and can pick the corresponding plaques.

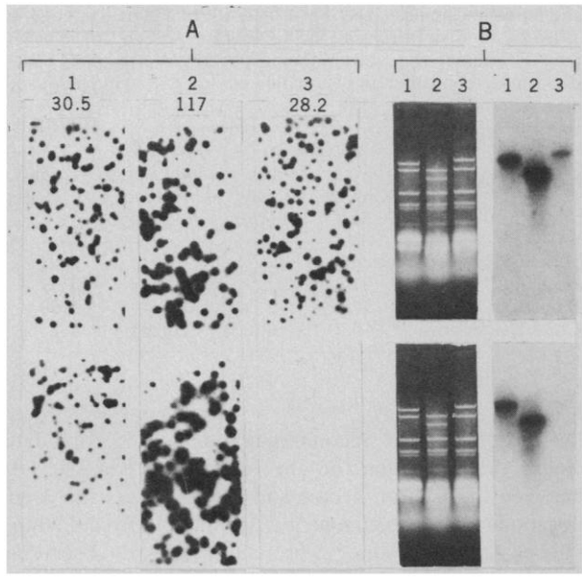


Fig. 5. Characterization of mouse genomic clones by competition hybridization. (A) Three phages, Mm30.5, Mm117Hbα, and Mm28.2, labeled 1, 2, and 3, respectively, were plated and two replicas of each were hybridized with a ^{32}P-labeled mouse globin cDNA probe (*24*). The top group was hybridized in the absence of poly(rA); the bottom group was hybridized with poly(rA). (B) Hpa I restriction digests of DNA from the same phages were prepared and subjected to agarose gel electrophoresis in two duplicate runs on a single gel (*29*). The ethidium bromide staining pattern of the gels is shown. Southern transfers (*27*) were made of the replicate gel portions, and each was hybridized with the ^{32}P-labeled mouse globin probe with (bottom) and without (top) poly(rA).

bacteriophage plaques in order to identify particular phages that contain homologous DNA sequences (*14*). Since plaques must be at least marginally separated to allow detection, it is necessary to allow about 2 to 4 cm^2 of lawn per 1000 phages screened. Our screening was scaled to the film (33 by 43 cm) used for medical x-rays.

Phages were plated on megaplates that were poured in aluminum foil-lined cafeteria trays (35 by 45 cm) (*22*). Large sheets of nitrocellulose were used to lift replicas. The DNA-DNA hybridization was done at 68°C in photography trays (*23*).

The first experiment to yield a genomic clone for a single copy gene also revealed that the mouse genome contains numerous clonable DNA segments that give very strong positive hybridization to radioactive polyadenylate [poly(A)] regions in the probes. In this experiment four megaplates (Nos. 28 to 31) from the mouse shotgun were screened. The autoradiogram from plate 30 is shown in Fig. 3A. A mixture of radioactive probes was used, including cDNA made from mouse α- and β-globin mRNA as well as nick-translated plasmid DNA's containing cDNA copies of rat insulin and rat growth hormone mRNA's (*24*). Forty-two positive hybridizing spots, including faint ones (Fig. 3A), were picked by the use of an alignment system (Fig. 4). All candidates were replated on standard petri dishes and rescreened by hybridization. Twenty-one candidates did not hybridize upon rescreening; these candidates correlated well with the faintest and most questionable spots on the original autoradiogram.

One of the candidates still hybridized specifically to the globin probe on retesting. This clone has been shown to contain α-type globin DNA (*5*) and has been designated Charon 3AMm30.5α, which can be abbreviated Mm30.5. Depending on the emphasis, this designation can be abbreviated by omitting any elements from the name but preserving their order.

The remaining 20 candidates on retesting hybridized both to globin probe and to a mixture of the three other probes. A few of these were tested further and shown to hybridize to all four probes individually. Since the only characteristic shared by the probes was their content of poly(A) regions, we suspected that A·T-rich (A, adenine; T, thymine) regions of the genome were being cloned. Mol *et al.* (*25*) have shown that the mammalian genome contains A·T-rich regions that can be isolated on oligo dT columns. Jeffreys and Flavell (*26*) have also included poly(rA) (riboadenylate) in their recent Southern hybridization (*27*) experiments to reduce the background of nonspecific hybridization. Therefore, to test the A·T hypothesis, competition hybridization experiments with poly(rA) were done by both plaque and Southern gel hybridization techniques. Figure 5 shows the results for one of the candidates, Mm28.2. Hybridization to the band containing the insert in Mm28.2, as well as hybridization to the plaques of this phage, could both be inhibited fully with poly(rA). All 20 of the questionable candidates were then tested for inhibition with poly(rA) by the plaque hybridization technique. All were inhibited, although hybridization to Mm30.5 and the control Mm117Hbα was unaffected by poly(rA). We consequently conclude that the hypothesis that these are clones of A·T-rich mouse DNA segments is supported.

Gel electrophoresis of Hpa I digests of eight of the clones of A·T-rich DNA was carried out. All had different-sized inserts. This shows that the A·T sequences occur in many contexts in the genome. We found that they could be avoided by including poly(rA) in the hybridization mixture.

In the search for human globin genes, we used nine master plates to screen a total of 3×10^6 phage plaques, of which approximately 1×10^6 contained fragments of genomic DNA. Five master plates from human shotgun L3, screened with mouse globin probe without poly(rA), yielded 107 globin candidates. None of these were globin-positive on retesting in the presence of poly(rA). Four other master plates, three from L3 and one from L2, were screened with human globin probe in the presence of poly(rA). They yielded ten candidates and, from these, one human clone, Hs51.1, was obtained (from shotgunL2). The autoradiogram from master plate 51 permitted the isolation of this human globin clone (Fig. 3B). It is evident (Fig. 3, compare A and B) that the addition of poly(rA) to the hybridization mixture improved the efficiency of the technique. It is possible that the use of probe from the same species also increases the probability of success, but we doubt it because both Hs51.1 and Mm30.5 hybridize well to both mouse and human probe.

To determine whether Mm30.5 and Hs51.1 are in fact clones of fragments of DNA that are present in the genome, Southern hybridization experiments were done. Eco RI was used to excise the hybridization positive DNA fragments from each of these clones. The fragments were partially purified on agarose gels, labeled with ^{32}P by nick translation (*24*), and used as probes for Southern hybridization to Eco RI–digested human or mouse DNA. The autoradiogram of the mouse experiment shows a prominent band of 4.7 kbp, the same size as the insert in Mm30.5 (*5*). In addition, when the autoradiogram was exposed for longer times, several weaker bands of 2.34, 5.25, 10.5, 12.0, 14.7, and 18.5 kbp were seen. Thus Mm30.5 is clearly a genomic clone of a 4.7-kbp fragment, but some sequences present in the cloned fragment are also present elsewhere in the genome. The experiment with Hs51.1 probe using human DNA also shows a band at the same position as the 2.7-kbp insert in Hs51.1 (*5*). In addition, a band of 7.4 kbp is seen. This band is

rougly equal in intensity to the 2.7-kbp band. No additional bands were seen with increased exposure of this autoradiogram. Therefore, we conclude that both Mm30.5 and Hs51.1 are genomic clones.

We have demonstrated the feasibility of cloning single copy genes from shotgun collections of phages constructed from unfractionated complete Eco RI digests of human and mouse DNA. Very little pure DNA is required for such experiments, and it need be manipulated only to the extent of digestion and ligation into the cloning vehicle. The key to obtaining this high efficiency is the use of in vitro packaging as the method for introducing recombinant DNA molecules into bacteria (*9–11*).

Two clones specific for globin sequences were isolated, one from each of the target genomes. Mouse globin cDNA is perfectly satisfactory for detecting plaques of the human globin clone Hs51.1, and human globin cDNA will detect the mouse globin clone Mm30.5. This principle will likely be useful in other cases for cloning the same gene from different species.

Our first approach was to screen each shotgun megaplate with several probes in the hybridization mixture. In this way we had hoped to isolate several interesting genes at once. Although this method is sound in principle, it is necessary that each probe used be free of anything that could hybridize nonspecifically. In many cases it may be simpler to screen the plates with each probe individually, since up to five nitrocellulose replicas can be taken from a single master plate. In this way, if any of the probes proves unsatisfactory, the whole experiment is not lost.

The major difficulty encountered has been the relatively common occurrence of simple sequence DNA's in the mammalian genome. Clones of A·T-rich DNA fragments yield hybridization-positive plaques when using probes containing stretches of poly(A) or poly(T) unless the hybridization is inhibited by poly(rA).

A second problem, of which we were aware at the outset, concerns the use of shotguns made from complete Eco RI digests. Such digests of mammalian DNA yield about 10^6 independent fragments, many of which contain only parts of the genes of interest. This causes problems in detecting clones corresponding to all the parts of desired genes, since some may not have sufficient DNA homologous to the probe to give a strong signal. The present type of shotgun collections are very simple to construct and use and are fine when the target is known to be on an Eco RI fragment smaller than 7 kbp. However, we are constructing new shotguns from DNA that contains partial as well as complete Eco RI digests, using our EK2 vector Charon 4A. This vector accepts inserts up to 22 kbp and will not grow with inserts less than 8 kbp. We expect that fewer clones will need to be screened with this strategy and that large (and small) gene fragments will be less likely to be missed.

The ability which we have demonstrated to isolate single copy mammalian genes from random shotgun collections of phages opens the way to the study of almost any gene from organisms of any complexity, provided that a reasonable probe can be obtained (*28*).

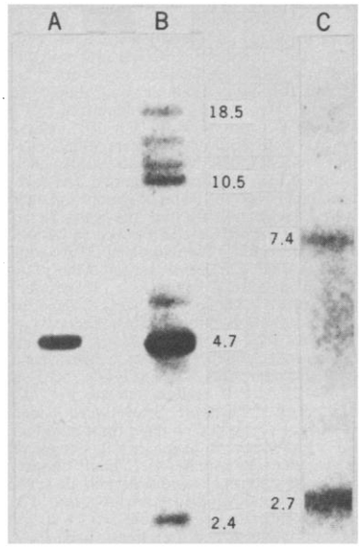


Fig. 6. Genomic identification of cloned fragments. Mouse and human DNA samples (25 μg) were completely digested with Eco RI and subjected to electrophoresis on 0.7 percent agarose gels. DNA was transferred to nitrocellulose filters by the Southern method (*27*) and hybridized. Radioactive probes were prepared by nick translation of partially purified cloned fragments that were isolated from the genomic clones by electrophoresis through 0.7 percent agarose gels. (A) ^{32}P-labeled 4.7-kbp fragment from Mm30.5 hybridized to mouse genomic DNA; the exposure time was 12 hours. (B) Same as (A), except that the exposure time was 40 hours. (C) ^{32}P-labeled 2.7-kbp fragment from Hs51.1 hybridized to human genomic DNA; the exposure time was 24 hours. Panel (C) has been photographically enlarged twofold to compensate for differences in the lengths of the gels.

FREDERICK R. BLATTNER
ANNE E. BLECHL
KATHERINE DENNISTON-THOMPSON
HARVEY E. FABER
JULIA E. RICHARDS
JERRY L. SLIGHTOM
PHILIP W. TUCKER
OLIVER SMITHIES

Laboratory of Genetics,
University of Wisconsin, Madison 53706

References and Notes

1. R. Higuchi, G. V. Paddock, R. Wall, W. Salser, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3146 (1976); T. Maniatis, S. G. Kee, A. Efstratiadis, F. C. Kafatos, *Cell* **8**, 163 (1976); F. Rougeon, P. Kourilsky, B. Mach, *Nucleic Acids Res.* **2**, 2365 (1975); T. H. Rabbitts, *Nature (London)* **260**, 221 (1976).
2. S. M. Tilghman, D. C. Tiemeier, F. Polsky, M. H. Edgell, J. G. Seidman, A. Leder, L. W. Enquist, B. Norman, P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4406 (1977); S. Tonegawa, E. Brack, N. Hozumi, R. Schuller, *ibid.*, p. 3518.
3. L. Clarke and J. Carbon, *Cell* **9**, 91 (1976); R. A. Kramer, J. R. Cameron, R. W. Davis, *ibid.* **8**, 227 (1976); K. Struhl, J. R. Cameron, R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1471 (1976).
4. L. H. Kedes, A. C. Y. Chang, D. Houseman, S. N. Cohen, *Nature (London)* **255**, 533 (1975).
5. O. Smithies, A. E. Blechl, K. Denniston-Thompson, N. Newell, J. E. Richards, J. L. Slightom, P. W. Tucker, F. R. Blattner, *Science* **202**, 1284 (1978).
6. *Fed. Regist.* **41**, 27902 (1975).
7. F. R. Blattner, *et al.*, *Science* **196**, 161 (1977); F. R. Blattner, D. O. Kiefer, D. D. Moore, J. deWet, B. G. Williams, Application for EK2 Certification of a Host Vector System for DNA Cloning, Supplement IX: "Data on Charon 21A."
8. H. E. Faber, D. Kiefer, F. R. Blattner, Application for EK2 Certification of a Host Vector System for DNA cloning, Supplement X: "Data on In Vitro Packaging Method."
9. A. Becker and M. Gold, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 581 (1975).
10. B. Hohn and K. Murray, *ibid.* **74**, 3259 (1977).
11. N. Sternberg, D. Tiemeier, L. Enquist, *Gene* **1**, 255 (1977).
12. H. F. Bunn, B. G. Forget, H. M. Ranney, *Human Hemoglobins* (Saunders, Philadelphia, 1977); D. J. Weatherall and J. B. Clegg, *The Thalassemia Syndromes* (Blackwell, Oxford, 1972).
13. S. M. Tilghman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4406 (1977); J. G. Mears, F. Ramirez, D. Leibowitz, A. Bank, *Cell* **15**, 15 (1978); J. G. Mears *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1222 (1978); Y. W. Kan, J. P. Holland, A. M. Dozy, S. Charache, H. H. Kazazian, *Nature (London)* **258**, 162 (1975).
14. W. D. Benton and R. W. Davis, *Science* **196**, 180 (1977).
15. N. Blin and D. W. Stafford, *Nucleic Acid Res.* **3**, 2303 (1976). Confluent monolayers of human fibroblasts (*16*) in four 500-ml roller bottles were rinsed with phosphate-buffered saline. Cells were lysed by 5 minutes of incubation in 50 ml of 1 percent Sarkosyl NL30, 0.1*M* EDTA, *p*H8, and proteinase K (100 μg/ml) at 56°C. Boiled ribonuclease (10 μg/ml) was added and the mixture was incubated at 56°C for 2 hours. The DNA fraction was extracted three times with a 1:1 mixture of phenol and chloroform–isoamyl alcohol (24:1, by volume). Solid CsCl was added to a final density of 1.70 g/cm^3, and the preparation was centrifuged for 60 hours at 30,000 rev/min (SW50.1 rotor). The DNA band was collected and dialyzed against 0.01*M* tris-Hcl, *p*H 7.0, 0.01*M* NaCl, 0.001*M* EDTA. Approximately 5 g of mouse liver, frozen in liquid nitrogen, was powdered by grinding under liquid nitrogen; then 50 to 100 ml of 0.1*M* EDTA, *p*H 8, with 1 percent Sarkosyl NL30 was heated to 50° to 55°C with magnetic stirring, and proteinase K (100 μg/ml) was added. The frozen liver powder was slowly added over a 1/2-hour period. Incubation at 56°C continued for 2 hours, and the DNA isolation was completed as above.
16. A human embryonic fibroblast culture was prepared by Dr. Robert DeMars in 1971 from a first trimester female abortus carefully dissected to free it from maternal cells, and it was divided in portions after one subculture in vitro and stored in liquid nitrogen. DeMars showed that the cells had a normal female karyotype, that they senesced after prolonged cultivation in vitro, and that contamination with maternal cells was below the limit of detection (10^{-3}) as measured by the fraction of azaguanine-resistant cells. (The mother was heterozygous for the Lesch-Nyhan mutation; the embryo was homozygous for normal alleles [J. S. Felix and R. DeMars, *J. Lab Clin. Med.* **77**, 596 (1971)].
17. H. Jacobsen and W. H. Stockmeyer, *J. Chem. Phys.* **18**, 1600 (1950); J. C. Wang and N. Davidson, *J. Mol. Biol.* **19**, 469 (1969); B. G. Williams, F. R. Blattner, R. K. Jaskunas, M. Nomura, *J. Biol. Chem.* **252**, 7344 (1977).
18. W. Mandel and A. Higa, *J. Mol. Biol* **53**, 159 (1970).

19. W. D. Henner, I. Kleber, R. Benzinger, *J. Virol.* **12**, 741 (1973).
20. Strain NS428, N100 (λ*A*am 1 1*b*2*red*3*c*I-857*S*am7), was a gift from N. Sternberg and L. Enquist. Strain dg805, W3350 (λdgal805*c*I-857*S*am7) was the gift from A. Becker. Strain NS428 was grown with shaking at 30° to 33°C in 600 ml NZY broth cultures (*7*) to A_{575} = 0.2, as measured on a Bausch and Lomb spectrophotometer (Spec 20). An additional 600 ml of 64°C NZY broth was added to the cultures to bring the temperature to 45°C, and the flask was incubated at 39°C with shaking for 1 hour (New Brunswick model G25). To test for successful induction a few milliliters of culture were shaken with chloroform and checked for the expected clearing. Freeze thaw lysate (FTL) was prepared by resuspending each pellet in 0.4 ml of cold 10 percent sucrose in 0.05*M* tris-HCl, *p*H 7.4, and transferring it to a 10-ml Oak Ridge type polycarbonate centrifuge tube. Egg white lysozyme (20 μl) (2 mg/ml, freshly dissolved in 0.25*M* tris-HCl, *p*H 7.4) was added with mixing, and the tubes were frozen in liquid nitrogen. Sonic extract (SE) was prepared by resuspending the NS428 pellet in 1.8 ml of buffer A (20 m*M* tris-HCl, *p*H 8, 3 m*M* $MgCl_2$, 0.05 percent 2-mercaptoethanol, 1 m*M* EDTA-KOH, *p*H 7), and was completely sonicated with cooling in an ice-salt bath until an opalescent solution free of intact cells was obtained. The preparation was centrifuged for 6 minutes at 4500*g*, and the supernatant was either used immediately or stored in liquid nitrogen. Protein A (pA) was prepared (A. Becker, personal communication) from six induced cultures of λdg805 prepared as described above for NS428, except that the heating was initiated at an absorbancy of 0.8. The induced cells were centrifuged and resuspended in 100 ml of buffer A and sonicated with cooling; the debris was removed by centrifugation. Polyethyleneimine (Miles, code 23-444) was dissolved to a final concentration of 10 percent in buffer A, and the solution was adjusted to *p*H 8 with HCl. This polyethyleneimine solution was added with stirring in the cold until no further precipitate formed (approximately 10 ml). The precipitate was collected by centrifugation and resuspended with an electric tissue homogenizer in 500 ml of 0.05*M* ammonium succinate (*p*H 6) containing 0.035 percent (by volume) 2-mercaptoethanol and centrifuged again; the pellet was resuspended in 500 ml of 0.1*M* ammonium succinate (as above) and centrifuged again. The pellet was resuspended in 0.2*M* ammonium succinate. The solution was again centrifuged, and the supernatant was saved. Neutralized ammonium sulfate (NAS) was prepared by adjusting saturated ammonium sulfate containing 0.1 percent 2-mercaptoethanol to *p*H 6.5; then 220 ml of NAS was added to the supernatant with stirring in the cold for 20 minutes. The precipitate was removed by centrifugation and 290 ml of NAS plus 15 g of solid ammonium sulfate was added to the supernatant. The precipitate was stirred for 30 minutes in the cold, collected, and resuspended in 5.5 ml of buffer A without $MgCl_2$ and dialyzed against the same buffer. One volume of glycerol was added, and the preparation was subdivided and stored in liquid nitrogen. Immediately before packaging, the FTL was thawed (<5°C) and held on ice for 45 minutes. Next, 0.05 ml of buffer M1 [6 m*M* tris-HCl (*p*H 7.4), 30 m*M* spermidine, 60 m*M* putrescine, 18 m*M* $MgCl_2$, 15 m*M* ATP (from a 0.1*M* stock at *p*H 7), 0.2 percent (by volume) 2-mercaptoethanol] was added, mixed, and centrifuged at 35,000 rev/min at 4°C for 25 minutes in a precooled type 65 rotor. Buffer A (30 μl) was mixed with 4 μl DNA (<1 μg), 4 μl buffer Ml, 20 μl thawed sonic extract, and 1 to 2 μl pA and incubated 15 minutes at room temperature. FTL (150 μl) was added and the mixture was incubated for 60 minutes at room temperature. The resulting phages were then plated.
21. To verify that the safety features of the Charon 3A vector were not compromised by the use of in vitro packaging, two tests were done on each batch of extracts as required by the NIH. (i) To determine whether endogenous DNA from the extracts was packaged, sham experiments were done in which no exogenous DNA was added to a 10× scale packaging mixture (2 ml final volume). The entire output was plated on 20 plates with lawns of bacteria permissive for the prophage in NS428. In five such experiments no phages were seen ($<1.5 \times 10^{-7}$ to $<7.5 \times 10^{-9}$ of the level that would be produced if vector DNA had been added). (ii) To determine whether in vitro packaging could result in loss of the amber mutations of the vector, 10× scale packaging experiments were done with vector DNA alone. In this case, the entire output was plated on a *su*° bacterial lawn to determine levels of amber$^+$ revertants. In five such experiments the highest level of reversion seen was 2×10^{-7}.
22. Cafeteria trays (35 by 45 cm) were lined with aluminum foil, sterilized with ethanol and ultraviolet and filled with 1.5 liters of bottom agar (*7*). After hardening in a still air hood, they were covered with sterile glass and transferred to P3 containment. Approximately 5×10^5 phages were mixed with 8 ml of stationary phase bacteria plus 8 ml of 0.01*M* $MgCl_2$ and 0.01*M* $CaCl_2$ and incubated at 37°C for 10 minutes. The mixture was then added to 150 ml of melted top agar (*7*), mixed, spread evenly over the warm, level surface of the megaplate and allowed to harden for 30 minutes before incubation at 37°C overnight. After development of the lawn the plate was chilled at least 12 hours in a refrigerator. The glass tops were lined with sheets of paper to reduce condensation. Nitrocellulose sheets type BA85 (33 by 45 cm) or rolls (33 cm by 3 m) (Schleicher and Schuell) were cut to size, rinsed in distilled water and 6× SSC (1× SSC is 0.15*M* NaCl, 0.015*M* sodium citrate, *p*H 7.2) and blotted on 3MM paper. These were placed on the surface of the chilled plate for 4 minutes. To aid in subsequent alignment, a series of marks spaced every 3 inches were made by penetration with a syringe needle dipped in India ink. The filter was then transferred to a bed of 3-MM paper saturated with 1.5*M* NaCl, 0.2*N* NaOH for 4 minutes and then neutralized for 4 minutes on a bed of the same paper saturated with 0.5*M* tris-HCl, *p*H 7.2, 3*M* NaCl, blotted and allowed to dry. Up to five filters can be lifted from a single plate with chilling between transfers. The nitrocellulose was then incubated between 3MM sheets for 2 hours at 80°C, and washed for 1 hour with gentle shaking at 68°C in 6× SSD (6× SSC, 0.02 percent Ficoll, 0.02 percent polyvinylpyrrolidone, 0.02 percent bovine serum albumin, 0.5 percent sodium dodecyl sulfate) [see D. T. Denhardt, *Biochem. Biophys. Res. Commun.* **23**, 641 (1966)].
23. The hybridization mixture contained poly(rA) (30 μg/ml; Sigma code P-8878), denaturated sonicated *E. coli* DNA (10 μg/ml) in 6× SSD. For each megaplate filter, 1×10^6 count/min of probe (*24*) was added to 300 ml of the mixture in a flat-bottomed photography tray. The tray was covered with a glass sheet, sealed within a plastic garbage bag, and incubated for 24 hours, with gentle shaking at 68°C. The filter was washed two or three times at 68°C (1 hour each) with shaking in 1 liter of 3 × SSC containing 0.5 percent sodium dodecyl sulfate, blotted, dried in air, and mounted for autoradiography beneath Kodak XR-1 film and a DuPont Quanta III intensifier screen. Exposure was for 2 days at −90°C. For Southern transfers of mini lysate gels (*27*), the hybridization procedure was the same, except that the concentration of probe was about 3×10^4 count/min per milliliter. For Southern transfers of genomic DNA gels (*26*), about 5×10^5 count/min per milliliter probe was used and hybridization was for 40 hours.
24. Human globin mRNA was provided by Drs. Arthur Bank and Francesco Ramirez and mouse globin mRNA was purified from mouse reticulocytes and provided by Dr. Jeffrey Ross. Mouse or human unfractionated mRNA was converted to [α-^{32}P]-labeled cDNA essentially by the method described [D. L. Kacian and J. C. Myers, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2191 (1976)]. The plasmids pRGH-1 [P. H. Seeburg, J. Shine, J. A. Martial, J. D. Baxter, H. M. Goodman, *Nature (London)* **270**, 486 (1977)] and pAU-1 [A. Ullrich, J. Shine, J. Chirgwin, R. Q. Pictet, E. Tischer, W. J. Rutter, H. M. Goodman, *Science* **196**, 1313 (1977)], containing rat growth hormone and rat insulin sequences, respectively, were a gift of Dr. Howard Goodman. The DNA's were made radioactive essentially according to the procedure described by T. Maniatis, A. Jeffrey, and D. G. Kleid [*Proc. Natl. Acad. Sci., U.S.A.* **72**, 1184 (1975)]. Double-stranded probes were denatured with NaOH.
25. J. N. Mol, R. A. Flavell, P. Borst, *Nucleic Acids Res.* **3**, 2367 (1967).
26. A. J. Jeffreys and R. A. Flavell, *Cell* **12**, 429 (1977).
27. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
28. *Note added in proof*: Similar work has just been published by T. Maniatis, R. C. Hardison, E. Lacey, C. O'Connell, D. Quon, G. K. Sim, A. Efstratiadis, *Cell* **15**, 703 (1978).
29. For rapid screening of clones by agarose gel electrophoresis of restriction digests, DNA was prepared directly from lysates. 0.4 ml SDS mix (0.25*M* EDTA, 0.5*M* tris-HCl, *p*H 9, 2.5 percent recrystallized sodium dodecyl sulfate) was added to 2 ml of clarified phage lysate and heated for 30 minutes at 70°C. At this point the samples contained no live phage and could be removed from P3 containment. Then 0.5 ml of 8*M* potassium acetate was added and the mixture was placed on ice for 15 minutes and centrifuged for 20 minutes at 12,000*g*. The supernatant was precipitated with two volumes of ethanol and centrifuged for 30 minutes at 27,000*g*. After the traces of ethanol were removed with a cotton-tipped swab, the pellet was dissolved in 0.4 ml of 0.3*M* sodium acetate, and precipitated again with ethanol. The pellet was dissolved in 50 μl of 1m*M* EDTA, *p*H 8; 1 to 5 μl of the preparation was digested with 2 units of restriction enzyme before agarose gel electrophoresis [T. M. Shinnick, E. Lund, O. Smithies, F. R. Blattner, *Nucleic Acids Res.* **2**, 1911 (1975)].
30. This is paper 2281 from the Laboratory of Genetics at the University of Wisconsin and paper 8 in the series, "Charon Phages for DNA Cloning." Supported by NIH grants GM21812 (F.R.B.), AM20120 and GM20069 (O.S.), CA09075 (K.D.T.), GM06526 and GM07131 (P.W.T.), and GM07133 (J.E.R. and A.E.B.); and an NIH research career development award (to F.R.B). We thank Howard Goodman for his collaboration with us in developing these procedures while visiting in our laboratory; Andrew Becker for hospitality in his laboratory; and D. O. Kiefer, N. Borenstein, T. C. Szeto, E. Kopetsky, and J. Kucera for technical assistance. This work was done under the NIH guidelines which require EK2, P3 containment.

12 October 1978

Cloning Human Fetal γ Globin and Mouse α-Type Globin DNA: Characterization and Partial Sequencing

Abstract. *Two globin-related clones isolated from collections of bacteriophages containing unfractionated Eco RI fragments of human and mouse DNA were characterized. Charon3AHs51.1Hbγ includes 2.7 kilobase pairs of human DNA containing a large part of a fetal γ globin chain structural gene; Charon 3AMm30.5 includes 4.7 kilobase pairs of mouse DNA related to α globin. The human fetal γ globin gene has within its coding region two intervening sequences of noncoding DNA, IVS 1 and IVS 2, of approximately 100 and 900 base pairs. Sequence IVS 1 is located at the position of one of the two intervening sequences occurring in adult globin genes; IVS 2 is located at the position of the other.*

Mammals utilize various α-type and β-type globin chains to make different hemoglobins at different stages of their lives. In humans, there are two α-type chains: the embryonic chain ζ, and the adult chain α; the β-type chains are the embryonic chain ε, the fetal chains ^{A}γ and ^{G}γ, the major adult chain β, and the minor adult chain δ (*1*). Various α- and β-type chains also occur at different stages in the development of mice (*2*). We hope to learn something about the control of

0036-8075/78/1222-1284$00.50/0