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Genetics

Isolation of transforming DNA by cosmid rescue

(recombinant DNA/yeast histone genes/restriction endonuclease map)

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ABSTRACT **A procedure has been developed to allow the recovery of an integrated plasmid genome from a transformed cell, together with large areas of the flanking DNA sequences. DNA from *Saccharomyces cerevisiae* BAS2, in which the pBR322–*ura* 3 plasmid (Y1p5) is integrated at the yeast histone H2A and H2B locus, was used to generate a cosmid library, using a new cosmid vector (pTL5) that is ampicillin sensitive and tetracycline resistant. Colonies were selected for ampicillin resistance, which was conferred by the incorporation of the integrated pBR322 β-lactamase gene into the recombinant cosmid. Restriction enzyme and blot hybridization analyses show that the rescued clones contain the yeast histone genes in addition to the Y1p5 sequences; a total of approximately 50 kilobase pairs of DNA sequences flanking the plasmid was recovered as a series of overlapping cosmids. This approach should allow the recovery of most genes that can be linked to a marker pBR322 sequence and for which a specific phenotype can be selected in a recipient eukaryotic cell.**

It is now commonplace to isolate genes by molecular cloning, following the traditional route of screening libraries with a nucleic acid probe for the desired gene. Most genes isolated by this approach are abundantly represented in mRNA populations. mRNAs for numerous cell functions, however, are present only in low copy numbers, say 5–10 copies per cell, because few molecules of their gene products are required. The cloning of the genes for these mRNAs by using the standard procedures is therefore difficult. In addition, there are several well-characterized genes for which the protein product and the mRNA have not been characterized at the molecular level. These considerations invite the development of cloning methods that circumvent the need for a nucleic acid probe for the gene required.

An alternative to the classical approach is to clone the gene by direct selection for the phenotype that it confers on a recipient cell. Many yeast genes have been cloned by this approach because they exhibit a demonstrable phenotype in *Escherichia coli*. This is unfortunately not true for the genes of most higher eukaryotes. To clone mammalian genes, therefore, an indirect approach must be followed. Perucho *et al.* (1) and Lowy *et al.* (2) transformed animal cells with a DNA preparation in which each molecule was physically linked to a bacterial plasmid. Because efficient methods have been developed for the stable genetic transformation of cultured animal cells, it is possible to select mammalian cells that have taken up genes that encode selectable markers, such as thymidine kinase, adenine phosphoribosyltransferase, and hypoxanthine phosphoribosyltransferase. In two cases, the desired genes have been recovered from the transformed cells, in the first by using the plasmid rescue technique (1) and in the second by constructing a phage library and screening for the plasmid DNA sequences by hybridization (2).

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We describe here an alternative procedure to plasmid rescue, based upon cosmid cloning (3), that permits the rescue of much larger genomic segments that contain a "transforming gene" linked to a selective marker that can be expressed in *E. coli*.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* HB101 (r_k^- m_k^- *leu pro recA*), *E. coli* ED8767 (*supE supF hsdS met recA58*), and *E. coli* DB6656 (*pyrF trp lacZ hsdR*) were obtained from D. Ish-Horowicz, K. Murray, and J. Beggs, respectively. *Saccharomyces cerevisiae* BAS2 and plasmid pY1p5-TRT-1 were kindly provided by L. Hereford; pHC79 (4) was obtained from J. Collins.

Isolation of DNA. Yeast DNA was isolated as described (5), except that the DNA was purified on a CsCl/ethidium bromide gradient prior to the RNase treatment.

Construction of the Cosmid Rescue Vector. See Fig. 1.

pTL1. pHC79 (50 μg) was digested with 60 units of *Pst* I and 50 units of *Eco*RI for 4 hr at 37°C, extracted with phenol, and precipitated with ethanol. The DNA was dissolved in 50 μl of 10 mM Tris·HCl (pH 7.5). Ten micrograms of the DNA (final concentration 200 μg/ml) was digested with 21 units of DNA polymerase I (Klenow fragment) in ligation buffer (6) containing all four deoxynucleoside triphosphates (25 μM) for 30 min at 37°C. The plasmid (20 μg/ml) was circularized by blunt-end ligation, using phage T4 ligase (2 hr at 37°C). The resulting plasmids were used to transform competent *E. coli* HB101 (7), and ampicillin-sensitive tetracycline-resistant colonies were picked.

pTL3. pTL1 (25 μg) was digested with 8 units of *Bal* I for 24 hr at 37°C, then digested with 5 units of *Hpa* I for 2 hr at 37°C. The fragments were separated by agarose gel electrophoresis and the required fragment was isolated by DEAE-cellulose chromatography, circularized by blunt-end ligation as above, and introduced into *E. coli* HB101 by transformation.

pTL4. pTL3 (36 μg) was digested with 9 units of *Bgl* II for 2 hr at 37°C, extracted with phenol, precipitated with ethanol, and taken up in 50 μl of 10 mM Tris·HCl (pH 7.5). The *Bgl* II-cut plasmid (25 μg) was digested with DNA polymerase I (Klenow fragment) and then circularized by blunt-end ligation as above. The resulting plasmids were introduced into *E. coli* HB101 by transformation.

pTL5. pTL4 (12 μg) and pHC79 (12 μg) were digested with 12 units of *Hin*dIII and 5 units of *Bst*EII (an isoschizomer of *Eca* I) for 90 min at 37°C, extracted with phenol, precipitated with ethanol, and taken up in 25 μl of 10 mM Tris·HCl (pH 7.5). Then 1.4 μg samples of the two digests were mixed and ligated at 250 μg of DNA per ml. The resulting plasmids were introduced into *E. coli* HB101 by transformation.

Cosmid Cloning. We used an adaptation of our cosmid cloning procedure (6) as developed by Ish-Horowicz and Burke (8). This modification is designed to give a high cloning efficiency

Abbreviation: kb, kilobase pair(s).

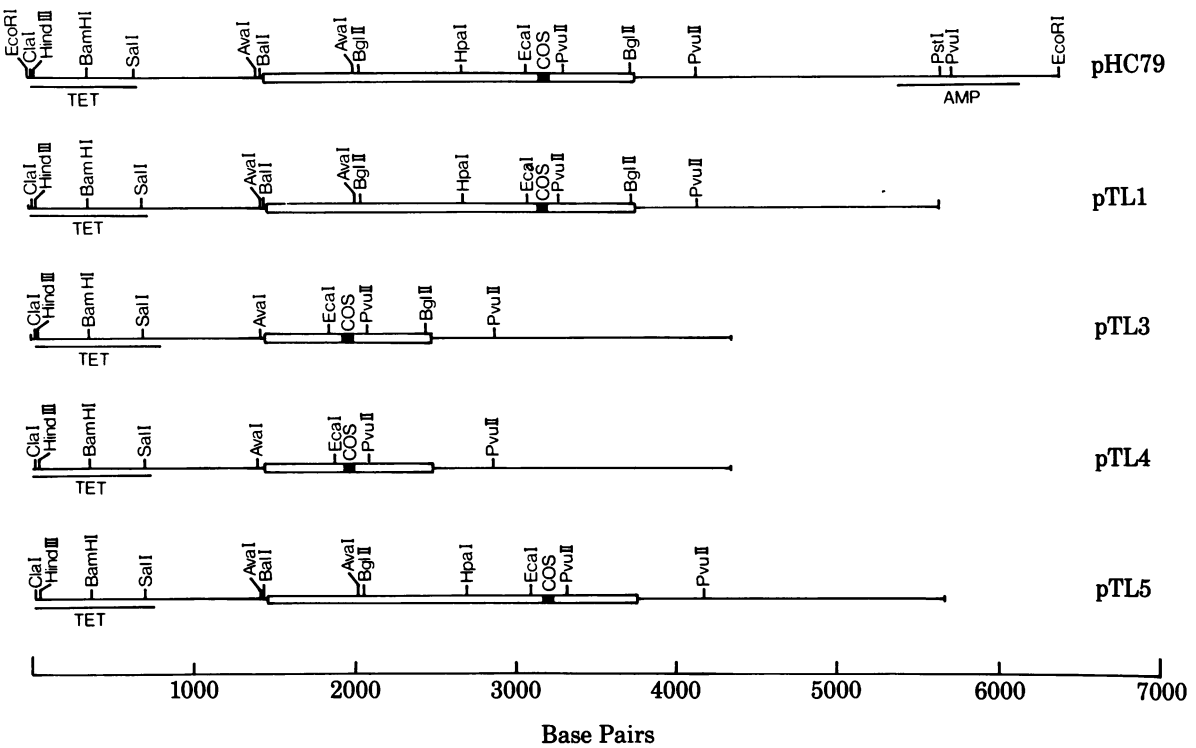

FIG. 1. Physical map of pHC79 (4) and the plasmids constructed to make the cosmid rescue vector pTL5. The double lines at TET (tetracycline resistance gene) and AMP (β-lactamase gene) indicate the approximate extent of the respective genes. COS refers to the λ phage cohesive end. Note that the isoschizomer *Bst*EII was used instead of *Eac* I in the construction and use of pTL5.

with a minimum of cosmids containing multiple inserts or vector molecules. The procedure is outlined in Fig. 2. pTL5 (25 μg) was digested with *Pvu* II (7 units) and *Bgl* II (7 units), extracted with phenol, and precipitated with ethanol. pTL5 (25 μg) was digested with *Bst*EII (7 units), extracted with phenol, and precipitated with ethanol. The *Bst*EII-cut plasmid was dissolved in 100 μl of 10 mM Tris·HCl (pH 7.5) and treated with 0.12 unit of calf intestinal phosphatase at pH 9.5 for 30 min at 37°C. The phosphatase was inactivated by heating at 65°C for 60 min and the DNA was extracted with phenol, precipitated with ethanol, dissolved in 200 μl of 10 mM Tris·HCl (pH 7.5) and digested with *Bgl* II (7 units). The *Pvu* II/*Bgl* II and *Bst*EII/*Bgl* II fragments were mixed (1:1 molar ratio) and ligated to partially digested *S. cerevisiae* BAS2 as follows: *S. cerevisiae* DNA (80 μg) was digested partially with *Mbo* I and fractionated by size as described (6). The DNA was pooled in four size classes: 30–40 kilobase pairs (kb), 20–30 kb, 10–20 kb, and <10 kb. The first three pools were used for rescue experiments. For this 1 μg of each pool was mixed with 1 μg each of *Bgl* II-cut pTL5 fragments, ligated, and packaged as described (6). The cosmids were transduced into *E. coli* ED8767, which was plated on either ampicillin or tetracycline plates, using the procedures described (6).

Enzymes. All enzymes were used under conditions recommended by the suppliers. Enzyme units are as defined by the suppliers. Southern blotting and filter hybridization were carried out according to standard procedures (9, 10).

RESULTS

Strategy of the Approach. The principle of the cosmid rescue procedure is outlined in Fig. 2. A eukaryotic cell is transformed with genomic DNA in which each DNA molecule is covalently linked to plasmid DNA that carries a selective marker such as the β-lactamase gene, either by ligating eukaryotic DNA with a linearized plasmid or by preparing DNA from a plasmid (11) or cosmid (6) library. In the latter case, it is certain that each eukaryotic DNA molecule is linked to the plasmid sequence; in the former this will be true for most of the molecules. After the transformed cells have been selected for the phenotype conferred by the newly acquired eukaryotic DNA, the cells are grown up and DNA is isolated. This DNA is cleaved under partial digestion conditions and 30- to 40-kb fragments, suitable for cosmid cloning, are obtained by size fractionation (Fig. 2). This DNA is then ligated to *Bgl* II-cut cosmid vector pTL5 and packaged *in vitro*. pTL5 was constructed as described in *Materials and Methods* and its map is shown in Fig. 1; its major features are a single *Bgl* II site for cloning *Mbo* I partial digests and the tetracycline resistance marker of pBR322 to allow selection of recombinant clones. Because the β-lactamase gene is partially deleted, recombinant clones are not ampicillin resistant unless the inserted DNA segment contains this gene. This generates a library of cosmid recombinants, all of which can be propagated in *E. coli* grown on tetracycline media. The DNA segments flanking the transforming gene will be present on a cosmid that can be propagated on both tetracycline (from pTL5) and ampicillin (from the integrated vector). Two options are available for the rescue experiment. The transforming gene can be rescued by plating on ampicillin media, or alternatively, by hybridization of the total clones from a tetracycline-resistant library with the 750-base pair *Pst* I/*Eco*RI fragment of pBR322, which is absent from pTL5 (Fig. 1). The sequences present on the rescued clones can then be characterized by standard methods. Here, we illustrate this method by rescuing the yeast *ura-3* and histone H2A and H2B genes from yeast chromosomal DNA.

Cosmid Rescue of the Yeast Histone Genes. Yeast strain BAS2, which contains the pBR322–*ura 3* plasmid Y1p5 integrated at the histone H2A and H2B locus, was constructed by transforming yeast with a histone H2A and H2B-gene–pBR322-*ura 3* plasmid (Y1p5-TRT-1) and selecting for *ura*-positive colonies (12). This generates a duplication of the histone genes in-

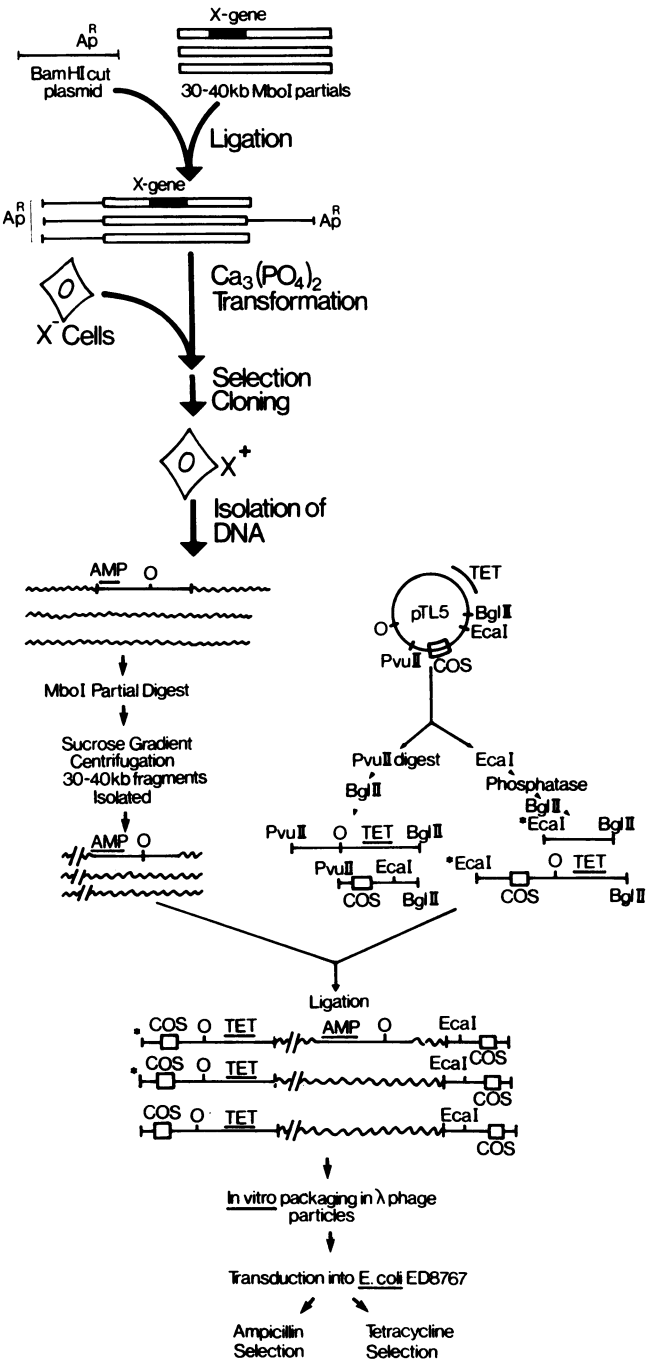

FIG. 2. (*Upper*) Scheme for the recovery of integrated plasmids by cosmid rescue. Ap^R denotes the β-lactamase gene. (*Lower*) Cosmid cloning procedure. As starting material, plasmid DNA carrying the β-lactamase gene (AMP) integrated into the genome of a eukaryotic cell is used as described in the text. COS is the phage λ cohesive end. TET denotes the tetracycline resistance gene of pTL5.

terrupted by the plasmid pY1p5 sequences (12). Fragments (30–40 kb) of *S. cerevisiae* BAS2 DNA obtained by partial digestion with *Mbo* I were ligated to the rescue cosmid pTL5, packaged *in vitro* and transduced into *E. coli* ED8767 as described in *Materials and Methods* and the mixture was plated on either tetracycline or ampicillin media. From 1.5 μg of yeast DNA, 16 colonies were obtained on ampicillin plates and approximately 18,000 on tetracycline. The ratio (colonies on ampicillin to colonies on tetracycline) is approximately the frequency expected for a single copy of the integrated ampicillin resistance plasmid per yeast haploid genome; all the ampicillin-resistant colonies were also resistant to tetracycline. DNA from each colony was characterized for the presence of the ampicillin resistance gene and the principal targets for the rescue experiment: the *ura 3* gene and the yeast histone H2A and H2B genes. The DNAs were tested for the *ura 3* gene by transforming *E. coli* DB6656 (*pyrF*) because the yeast *ura 3* gene complements this *E. coli* auxotroph (13). DNA from 13 of the 16 rescued clones conferred growth on minimal media lacking uracil.

The presence of the β-lactamase of pBR322 and the λ phage cos end was shown by hybridizing Southern blots of *Bam*HI-cut rescued cosmids with the *Pst* I/*Eco*RI fragment of pBR322 and the 1.6-kb *Bgl* II λ phage cos fragments from pHC79, respectively, as probes (data not shown). In all cosmid clones a single band hybridized with each probe, showing the presence of both components. According to the physical map of the integrated pY1p5, a 5.4-kb fragment should be obtained with the pBR322 probe; this is seen in 10 of the clones.

To detect the yeast histone H2A and H2B genes, clones pRY2, -3, -7, -13, and -15 were cut with *Bam*HI or *Eco*RI, electrophoresed in agarose gels, blotted, and hybridized with the 6.4-kb fragment of the yeast H2A and H2B genes (5). Yeast chromosomal DNA from *S. cerevisiae* BAS2 shows two *Eco*RI fragments, 10.0 kb and 11.5 kb, that hybridize with the histone gene probe (Fig. 3). These fragments are seen in clones pRY1 (not shown) and pRY2 (Fig. 3); clones pRY6 (not shown), -7, -13, and -15 (Fig. 3) contain the 11.5-kb fragment and clones pRY3 (Fig. 3), -4, and -8, (not shown) contain only the 10.0-kb fragment. This suggests that the former clones extend to the left side of the histone gene map of Fig. 5 and therefore lack the right-hand 10.0 kb; conversely, clones pRY3, -4, and -8 should extend to the right-hand side of the map of Fig. 5 and lack the intact left-hand 11.5-kb fragments.

This interpretation is supported by the analysis of *Eco*RI digests of these cosmids. In Fig. 4 the clones are aligned in order, going from left to right in the map of Fig. 5. It can be seen that the clones contain a series of overlapping *Eco*RI fragments, and these fragments can be ordered (Fig. 5) on this basis. Thus, clones extending 18.5 kb to the left-hand side (called LH clones) of the 11.5-kb histone-gene fragment all contain several common *Eco*RI fragments. Clone pRY2, which contains both the 10.0-kb and the 11.5-kb histone gene fragments, serves as a reference for the comparison of the other clones. It contains the 1.5- and 0.8-kb fragments also present on the LH clones of Fig. 5; these also contain the 11.5-kb histone gene fragment, but not the 10.0-kb fragment. In addition, a 2.2-kb *Eco*RI fragment is

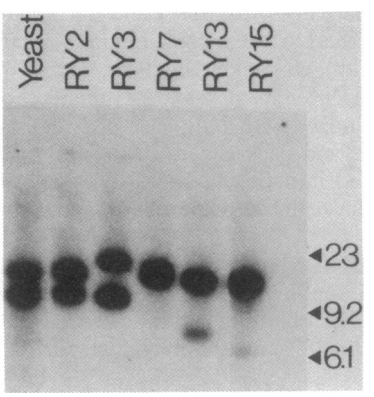

FIG. 3. Detection of the 6.4-kb histone fragment in some of the rescued clones. Yeast BAS2 DNA (2 μg) and clones pRY2, pRY3, pRY13, and pRY15 (2 ng each) were digested with *Eco*RI. The DNA was separated on a 0.5% agarose gel, denatured *in situ*, transferred to a nitrocellulose filter, and hybridized with a ^{32}P-labeled histone gene fragment from TRT-1 (5). The positions of marker fragments, with lengths given in kb, are indicated on the right, as they are in Figs. 4 and 6.

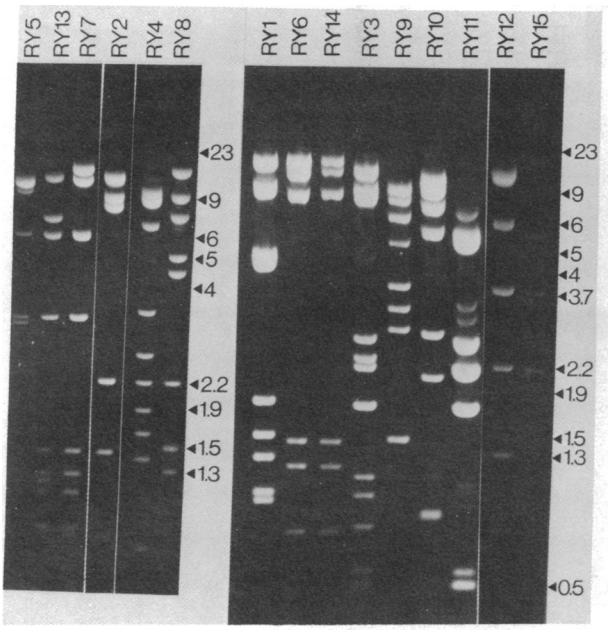

FIG. 4. Analysis of the rescued cosmid clones aligned in the order as proposed in the map in Fig. 5. All clones were digested to completion with *Eco*RI, and the fragments were separated on a 0.5% agarose gel. The clones pRY1, -6, -14, -3, -9, -10, -11, -12, and -15 are not colinear with the physical map shown in Fig. 5 and may either have been derived from rearrangement of the DNA sequences during transformation or consist of several *Mbo* I fragments linked during the ligation. It should be noted that all of these clones contain at least part of the histone genes and that in many cases (e.g., pRY6 and pRY14) have other *Eco*RI fragments in common with the colinear clones.

visible, which is absent in these LH clones but present in clones pRY1, -4, -8, and -15. This suggested that this fragment was present on the right-hand side of the map (Fig. 5); the 2.2-kb fragment was therefore isolated and hybridized to *Eco*RI, *Hin*dIII, *Cla* I, and *Pst* I digests of these clones and of genomic DNA. Fig. 6 shows that these clones all hybridize to the 2.2-kb fragment except pRY15 (not shown), which contains a 2.2-kb *Eco*RI fragment that is not homologous to the probe. This experiment identifies the *Eco*RI and *Hin*dIII sites of this region. All clones that hybridize with the 2.2-kb *Eco*RI probe show the 3.6-kb *Hin*dIII fragment, seen also in genomic DNA, except pRY4, which has an 8.1-kb *Hin*dIII fragment. We conclude that the colinear yeast DNA insert of this clone terminates between the *Eco*RI and *Hin*dIII sites of this region of Fig. 5. By the same arguments, genomic DNA and clones pRY1 and pRY8 contain a 5.6-kb *Pst* I fragment complementary to the 2.2-kb *Eco*RI probe; clones pRY2 and pRY4, however, both have larger fragments, and we conclude that these clones terminate before the distal *Pst* I site in this region. Finally, the colinear segments of the yeast DNA in the remaining clones terminate before the *Cla* I site present in genomic DNA, because none of the clones exhibit the same fragment seen with genomic DNA. These clones contain additional yeast DNA that has not been mapped in detail. It may be a noncolinear segment fortuitously attached to the histone region during the ligation step or due to rearrangements in this region in the cosmid DNA during, or subsequent to, the cloning step. This point has not been investigated.

The rescued clones pRY1, -3, -6, -9, -10, -11, -12, -14, -15, and -16 all contain the β-lactamase gene of pY1p5 (data not shown) and in addition the 11.5-kb *Eco*RI histone fragment (pRY1, -6, and -14), the 10.0-kb *Eco*RI fragment (pRY1, -3, and -10), or truncated fragments of histone genes (pRY11, -12, -15, and -16) (results not shown). However, none of these clones contain DNA fragments that are colinear with the fragments indicated on the map (Fig. 5), suggesting that the sequences flanking the plasmid or histone genes may be derived either from rearrangement of DNA sequences during *in vitro* packaging and transformation or from linkage of two or more *Mbo* I fragments during ligation, because many of these clones derived from yeast DNA fragments smaller than 30 kb.

DISCUSSION

The aim of the rescue experiment described here was to recover a large segment of DNA containing the histone H2A and H2B locus of *S. cerevisiae*, using integrated plasmid pY1p5 sequences as a marker. The rescued clones obtained contained all the expected DNA sequences—the β-lactamase gene of pBR322, the yeast *ura 3* gene, and the yeast histone H2A and H2B genes. In addition, in several clones considerable areas of the flanking DNA regions were recovered.

Although several of the rescued clones can be aligned into a series of overlapping clones derived from the histone gene region, this was not true in all cases. The aberrant clones that do not appear to be colinear with DNA from the histone gene region may be the result of rearrangement of DNA sequences after cloning or the result of the joining of shorter DNA sequences during ligation. Whatever the explanation for this phenomenon, it is clear that it does not prevent the recovery of the selected genes. It does, however, emphasize the necessity of checking the rescued DNA with its corresponding genomic region by phenotypic analysis and Southern blotting. The latter is, in general, required for all cloned sequences, because rearrangements have been shown to occur in genomic DNA cloned in *E. coli* by standard procedures (14, 15).

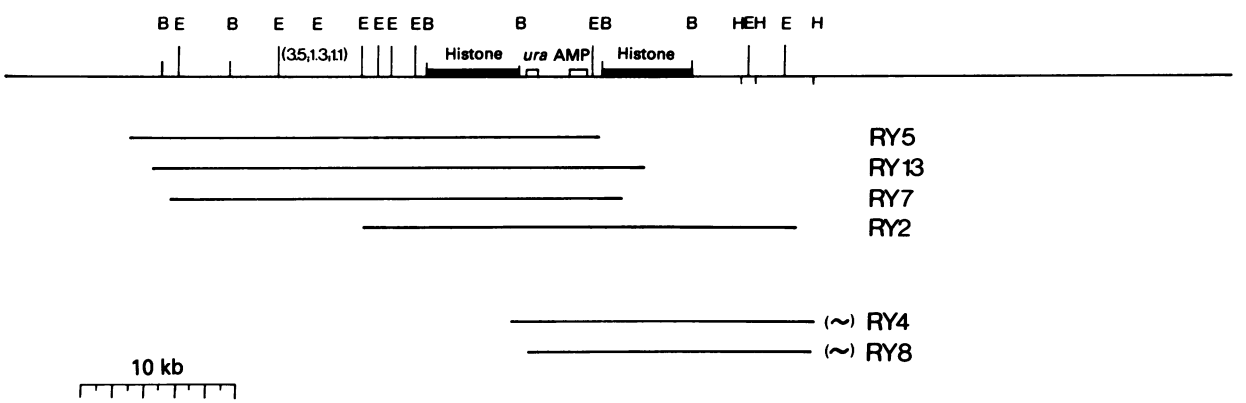

FIG. 5. Physical map of *Eco*RI (E) and *Bst* I (B) restriction endonuclease cleavage sites in and around the integrated pY1p5-TRT-1 DNA and the location of some of the rescued clones containing the histone genes. Only the *Hin*dIII (H) sites around the 3′ 2.2-kb *Eco*RI fragment are indicated. Clones pRY4 and pRY8 contain additional DNA (~) that is not colinear with the yeast DNA of this region.

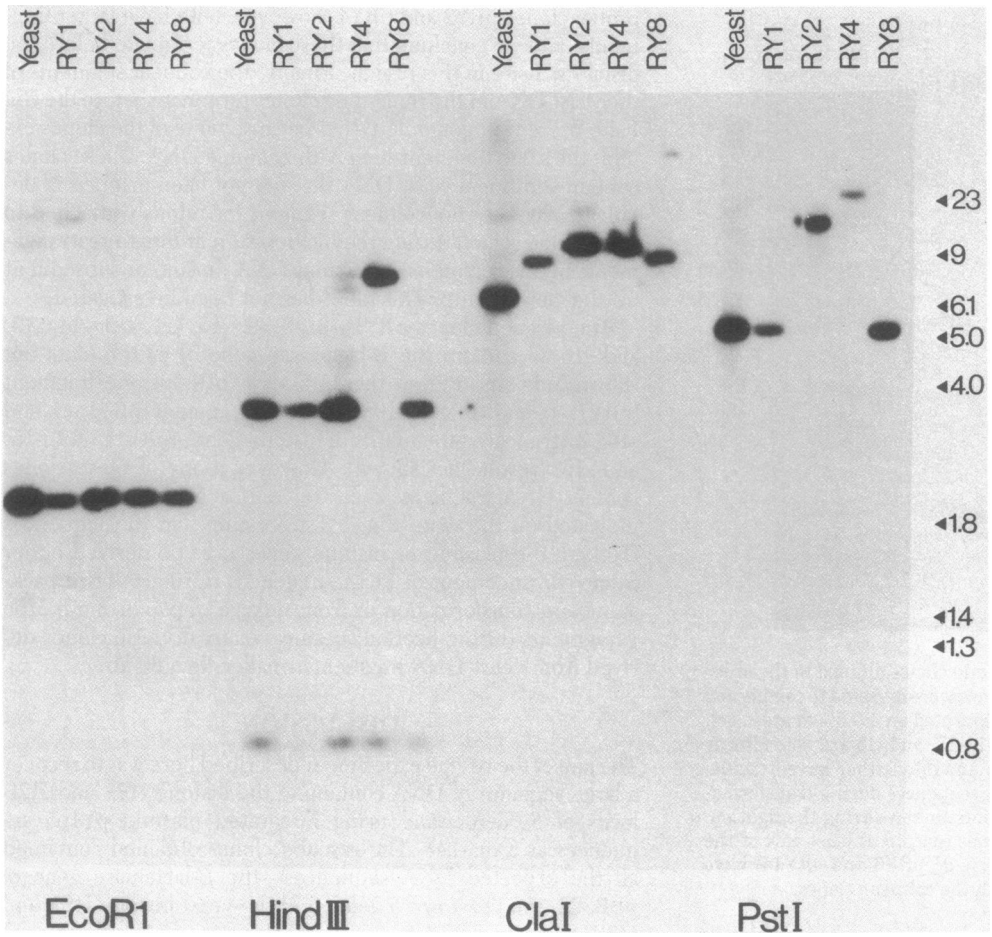

FIG. 6. Localizing the 3′ boundary of the restriction nuclease map in Fig. 5. Yeast BAS2 DNA (1 μg) and pRY1, -2, -4, and -8 (1 ng each) were digested with *Eco*RI, *Hin*dIII, *Cla* I, and *Pst* I. The DNA was separated on a 0.5% agarose gel, transferred to a nitrocellulose filter by the Southern technique, and hybridized with a ^{32}P-labeled 2.2-kb *Eco*RI fragment of pRY2.

Cosmid rescue should be applicable to any integrated DNA segment for which a selection can be applied in both the prokaryotic and the eukaryotic phase. Although the efficiency of plasmid rescue in the studies reported was about 1/100th of that expected (1), the frequency of recovery of the desired DNA segment by cosmid rescue is approximately that expected for a single-copy gene. Because the rescued DNA segments are 30–40 kb in length, it should be possible to recover large genes intact or extensive regions in or around a given smaller gene. The method described here should permit the use of DNA derived from cosmid libraries such as described by Grosveld *et al.* (6) to transform a eukaryotic cell to a given new phenotype and subsequently to rescue the DNA sequences responsible for the new phenotype by this procedure.

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1. Perucho, M., Hanahan, D., Lipsick, L. & Wigler, M. (1980) *Nature (London)* **285**, 207–210.
2. Lowy, I., Pellicer, A., Jackson, J. F., Sim, G.-K., Silverstein, S. & Axel, R. (1980) *Cell* **22**, 817–823.
3. Collins, J. & Hohn, B. (1979) *Proc. Natl. Acad. Sci. USA* **75**, 4242–4246.
4. Hohn, B. & Collins, J. (1980) *Gene* **11**, 291–298.
5. Hereford, L., Fahrner, K., Woolford, J., Rosbach, H. & Kaback, D. B. (1979) *Cell* **18**, 1261–1271.
6. Grosveld, F. G., Dahl, H. H. M., de Boer, E. & Flavell, R. A. (1981) *Gene* **13**, 227–237.
7. Kushner, S. (1978) in *Genetic Engineering*, eds. Royer, H. W. & Nicosia, S. (Elsevier, North–Holland, Amsterdam), pp. 17–28.
8. Ish-Horowicz, D. & Burke, J. F. (1981) *Nucleic Acids Res.* **9**, 2989–2998.
9. Flavell, R. A., Kooter, J. M., de Boer, E., Little, P. F. R. & Williamson, R. (1978) *Cell* **15**, 25–41.
10. Bernards, R. & Flavell, R. A. (1980) *Nucleic Acids Res.* **8**, 1521–1534.
11. Clarke, L. & Carbon, J. (1976) *Cell* **9**, 91–99.
12. Osley, M. A. & Hereford, L. M. (1981) *Cell* **24**, 377–384.
13. Chevallier, M. R., Block, J. C. & Lecraute, F. (1980) *Gene* **11**, 11–19.
14. Lauer, J., Shen, C. K. J. & Maniatis, T. (1980) *Cell* **20**, 119–130.
15. Fritsch, E. F., Lawn, R. M. & Maniatis, T. (1980) *Cell* **19**, 959–972.