The development of new strategies for the in vivo modification of eukaryotic genomes has become an important objective of current research. Site-specific recombinases have proven useful, as they allow controlled manipulation of murine, plant, and yeast genomes. Here we provide the first evidence that the prokaryotic site-specific recombinase (\(\beta\)-recombinase), which catalyzes only intramolecular recombination, is active in eukaryotic environments. \(\beta\)-Recombinase, encoded by the \(\beta\) gene of the Gram-positive broad host range plasmid pSM19035, has been functionally expressed in eukaryotic cell lines, demonstrating high avidity for the nuclear compartment and forming a clear speckled pattern when assayed by indirect immunofluorescence. In simian COS-1 cells, transient \(\beta\)-recombinase expression promoted deletion of a DNA fragment lying between two directly oriented specific recognition/crossing over sequences \(\langle\text{six sites}\rangle\) located as an extrachromosomal DNA substrate. The same result was obtained in a recombination-dependent \(\beta\) activation system tested in a cell line that stably expresses the \(\beta\)-recombinase protein. In stable NIH/3T3 clones bearing different number of copies of the target sequences integrated at distinct chromosomal locations, transient \(\beta\)-recombinase expression also promoted deletion of the intervening DNA, independently of the insertion position of the target sequences. The utility of this new recombinase tool for the manipulation of eukaryotic genomes, used either alone or in combination with the other recombination systems currently in use, is discussed.

Several methods have been developed allowing the manipulation of mammalian genomes in order to elucidate the relevance and function of particular genes of interest. Among them, the development of transgenic mouse strains and gene targeting technologies has been particularly useful \(\langle1, 2\rangle\). These techniques have experienced a new advance with the characterization and application of site-specific recombinases \(\langle3\rangle\).

**The Prokaryotic \(\beta\)-Recombinase Catalyzes Site-specific Recombination in Mammalian Cells**

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Site-specific recombinases can be clustered into two major families. The Int family comprises those enzymes that catalyze recombination between sites located either in the same DNA molecule (resolution and inversion) or in separate DNA molecules (integration) \(\langle4-7\rangle\). The latter property has been exploited to allow targeted insertion of specific sequences at precise locations \(\langle8, 9\rangle\). The recombinases currently used to manipulate mammalian genomes are mainly the Cre and Flp proteins, both members of the Int family \(\langle3\rangle\). The target sequences for these enzymes, \(\text{loxP}\) sites for the Cre enzyme and FRT for Flp, consist of a short inverted repeat to which the protein binds. The recombination process is operative through long distances \(\langleup to 70 \text{ kilobases}\rangle\) in the genome. Using these enzymes, several authors have reported site- and tissue-specific DNA recombination in murine models \(\langle10-13\rangle\), chromosomal translocations in plants and animals \(\langle14-16\rangle\), and targeted induction of specific genes \(\langle17\rangle\). For instance, expression of Cre from the \(\text{lck}\) proximal promoter leads to specific recombination in thymus \(\langle10\rangle\). The gene encoding DNA polymerase \(\beta\) has been tissue-specifically deleted using the same strategy \(\langle11\rangle\). In a different approach, the SV40 tumor antigens have been specifically activated in the lenses of mice, resulting in tumors at that location and not in the rest of the animal \(\langle17\rangle\). The Cre-\(\text{loxP}\) strategy has also been used in combination with inducible promoters, as in the case of an interferon-responsive promoter that was used to provoke gene ablation in liver with high efficiency and, to a lesser extent, in other tissues \(\langle12\rangle\).

The second family of recombinases includes those enzymes that catalyze recombination only when the sites are located in the same DNA molecule \(\langle\text{resolution and/or inversion}\rangle\); they are collectively termed resolvases/invertases \(\langle18\rangle\). \(\beta\)-Recombinase, which belongs to this family, catalyzes exclusively intramolecular deletions and inversions of DNA sequences located between two target sites for the recombinase, called \(\text{six sites}\) \(\langle19, 20\rangle\). Each \(\text{six site}\) comprises 90 bp \(\langle\text{see Eq. 1}\rangle\) and is composed of two subsites, termed I and II, to which the recombinase binds \(\langle19, 21\rangle\). \(\beta\)-Recombinase is encoded by the \(\beta\) gene of the Gram-positive broad host range plasmid pSM19035 \(\langle22, 23\rangle\). In this study, we have explored the utility of the prokaryotic site-specific \(\beta\)-recombinase for the manipulation of mammalian genomes. We describe the cloning and expression in eukaryotic cells of the gene coding for \(\beta\)-recombinase and show its ability to catalyze site-specific resolution \(\langle\text{deletion}\rangle\) of DNA sequences when the target sequences are either in a plasmid \(\langle\text{extrachromosomal target}\rangle\) introduced into the cell by transfection or integrated in the genome as chromatin-associated structures at several locations. The possible applications and potential advantages of this new system, specifically in combination with those already in use, are discussed.

\[1\text{ The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction.}\]

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**EXPERIMENTAL PROCEDURES**

Plasmids and Cloning—Plasmids pBT338 and pCB6, carrying either one or two directly oriented six sites (19), and pLXSN, which carries the resistance marker for neomycin (G418) (24), have been previously described. A eukaryotic expression vector with the SV40 early promoter, pSV2 (25), was kindly provided by Dr. J. Ortin (Centro Nacional de Biotecnologia). The expression plasmid pSVj2 was constructed by PCR amplification of the coding sequence for the β gene from plasmid pBT233 (22). The primers used for PCR were as follows: betaUP, 5′-GAGAGAAGGCTTTGTTGAAAATGGCT-3′; and betaDO, 5′-GAGAGATGATCAGTACTCATTAACTATCCC-3′. These oligonucleotides contain restriction sites for HindIII and BclI, respectively, which were used to clone the amplified gene in the pSV2 vector following standard methods (26). Since BclI is sensitive to methylation, the pSV2 plasmid was isolated from the BZ101 (dam-1) bacterial strain. The relevant structures are depicted in Fig. 1.

Culture and Cell Lines—Transient expression assays were performed in the simian COS-1 cell line, kindly provided by Dr. J. Ortin. Stable clones with the DNA substrate for β-recombinase integrated at different chromatin sites were established in the murine cell line NIH/3T3. Both cell lines were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. 

Different chromatin sites were established in the murine cell line NIH/3T3. Both cell lines were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum.

β-galactosidase detection was performed on Western blots as described. A monoclonal anti-β-actin antibody (Sigma) was used as a loading control. The presence of nuclear fraction proteins was monitored with a monoclonal anti-histone antibody (Chemicon International, Inc., Temecula, CA). Peroxidase-conjugated anti-mouse IgM antibody (Dako) was used as secondary antibody for both purposes.

**RESULTS**

Expression of the Prorhodacytotic β-Recombinase in Mammalian Cells—The coding sequence for β-recombinase was cloned in the pSV2 vector under the control of the SV40 early promoter. A control plasmid that does not contain the β-gene was also generated during this process (pSVc). The resulting constructs, pSVβ2 (Fig. 1B) and pSVc, respectively, were transiently transfected in COS-1 cells, which express SV40 T-antigen. Under these conditions, the expression from plasmids that contain the SV40 early promoter (included in pSVβ2) is amplified.

Transiently transfected cells were stained with rabbit polyclonal anti-β-recombinase antibodies. Fluorescence microscopy of the pSVβ2-transfected cells showed a strong speckled signal located specifically in the cell nucleus (Fig. 2, D and E). However, very faint staining was detected in the mock and control (pSVc) samples. Western analysis confirmed the presence of nuclear fraction proteins was monitored with a monoclonal anti-histone antibody (Chemicon International, Inc., Temecula, CA). Peroxidase-conjugated anti-mouse IgM antibody (Dako) was used as secondary antibody for both purposes.
Similar results were obtained when expression was tested by immunoblotting (Fig. 2F). A specific 25-kDa band, with a mobility corresponding to that of purified β-protein (Fig. 2F, c lane), was developed by the anti-β-recombinase antibodies when COS-1 cells were transfected with the pSVβ2 plasmid (+ lane), but not in the mock-transfected cells (− lane). Definitive evidence for the preferential nuclear location of β-recombinase was provided by subcellular fractionation experiments. As shown in Fig. 3, the specific band corresponding to β-recombinase appeared only on the nuclear enriched fraction of the pSVβ2-transfected cells.

These results indicate that β-recombinase can be expressed in eukaryotic environments, showing strong avidity for the nuclear compartment. Additional experiments with stable β-recombinase-expressing clones showed the same cellular distribution, without affecting cellular viability.3

β-Recombinase Catalyzes Site-specific Recombination in Transiently Transfected Mammalian Cells— Unlike integrases with simple recombination sites, such as Cre and Flp, which

3 V. Díaz, F. Rojo, C. Martínez-A., J. C. Alonso, and A. Bernad, unpublished results.
catalyze inter- and intramolecular recombination and do not require additional protein factors (4, 5, 7). β-recombinase catalyzes intramolecular recombination and has a strict requirement for a chromatin-associated protein to mediate DNA recombination (19, 20). β-Recombinase binds to the six sites and, with the help of a chromatin-associated protein, promotes strand exchange (Fig. 1A). The accessory factor is a chromatin-associated protein such as prokaryotic HU or eukaryotic HMG1 protein (20, 28, 29).

To determine whether eukaryotic cells could provide this host factor, recombination activity due to β-recombinase was first tested by transient cotransfections in COS-1 cells with plasmids pSVβ2 (bearing the β-recombinase gene) and pCB8 (the substrate DNA containing two target sites for β-recombinase in direct orientation flanking the xyle gene; see Fig. 1B). Upon recombination, two derivatives of pCB8, with a single six site each, should be obtained. The presence of one of these recombination products can be easily monitored by PCR amplification of Hirt extracts using primers complementary to the sequences located upstream of one of the six sites (primer a in Fig. 1B and under “Experimental Procedures”) and downstream of the second six site (primer b in Fig. 1B and under “Experimental Procedures”). In pCB8, these two primers hybridize to sequences located >2.7 kilobases apart. Under our PCR conditions, this fragment was not efficiently amplified; nevertheless, a 555-bp DNA segment should be amplified from the recombination product. A band of similar length should be obtained when using the same primers and plasmid pBT338 as template, which contains a single six site and was used as positive control (Fig. 1B). After transfection of the COS-1 cells (48 h), the extrachromosomal fraction (Hirt extraction) of the cells was therefore purified, and the presence of recombination products was analyzed by PCR. An amplified band of the expected length (555 bp) was observed only when both pCB8 and pSVβ2 plasmids were cotransfected (Fig. 4); this band was absent when the two DNAs were transfected separately or when pCB8 was cotransfected with pSVc, the negative control plasmid. The specificity of the amplified band was further confirmed by Southern hybridization (Fig. 4, lower panel) with a probe specific for the six site (see Fig. 1A). A positive signal of the correct size was detected only in the positive control lane (Fig. 4, pBT338 (+)) and in the pCB8/pSVβ2 cotransfection sample. In lanes corresponding to transfections containing the pSVβ2 plasmid, the additional band of smaller size detected on the agarose gel was demonstrated to be nonspecific, as it did not hybridize to the probe containing the six site sequence. These results indicate that β-recombinase is active in a eukaryotic environment, using the machinery/factors provided by the host cell.

To provide further experimental evidence of the β-recombinase-mediated process in eukaryotic cells, a new set of vectors was constructed for recombination-activated gene expression (Fig. 5A). The assay vector consisted of the lacZ gene separated from the SV40 early promoter by the pac gene (which confers resistance to puromycin in bacteria and eukaryotic cells) flanked by two six sites in direct orientation. Upon recombination, the pac gene should be excised from the plasmid, leaving the lacZ gene under the control of the SV40 promoter, thus rendering expression of β-galactosidase activity. This reporter gene can easily be monitored and quantified in cell extracts. The negative control (plasmid pPursxgal) lacks the first six site and is not a suitable substrate for recombination. A positive control (plasmid pgal) was obtained by in vitro recombination (19) of the Recombiner plasmid using purified β-recombinase and further isolation and characterization.

Upon transfection of these plasmids in a stable β-recombinase-expressing cell line, the whole protein fraction was extracted from each condition and assayed for β-galactosidase activity. As shown in Fig. 5B, transfection of the Recombiner construct promoted β-galactosidase expression several orders of magnitude higher than the mock and pPursxgal transfection.
The proteins were extracted, and are indicated. Each transfection was performed in triplicate. After 48 h, several stable clones were analyzed by Southern hybridization. NIH/3T3 cell clones in which the pCB8 construct was integration of the mean cpm ± S.D. from each triplicate condition. Equivalent transfection experiments on the parental cell line not expressing β-recombinase rendered no detectable β-galactosidase activity, demonstrating β-recombinase dependence of the measured activity. However, the β-galactosidase activity induced by transfection of the Recombiner construct was not in the same range as the one obtained with the positive control (pgal transfection). One plausible reason for this result could be that recombination occurs in a time period close to that used in the experimental conditions. Since pgal is already recombined, expression of β-galactosidase from this plasmid occurs early after transfection. This is not the case of Recombiner, which has to become recombined prior to lacZ gene expression. As a result, the number of cells with recombined plasmid is less in Recombiner transfection 48 h later than in pgal transfection, and therefore, β-galactosidase accumulation is reduced.

**DISCUSSION**

The common genome manipulation techniques, including transgenesis and gene targeting, have opened a new path for the understanding of a wide variety of mechanisms involving diverse genetic functions. The utility of these systems becomes limited, however, when the overexpression or inactivation of a given gene has fatal effects on embryo development (as an example, see Refs. 11 and 30) or when the lack of gene function can be bypassed or compensated by redundant mechanisms (31, 32). Moreover, the effects of gene inactivation outside the tissue or cell lineage of interest are usually unknown and uncontrollable (33).

These problems have been overcome to some extent by the development and application of the site-specific recombination techniques (reviewed in Ref. 7) that allow spatiotemporal control of the targeting event. This is the case of the Cre-loxP and Flp-FRT systems (reviewed in Refs. 3 and 4).

We show that the prokaryotic β-recombinase, which belongs to the resolvase/invertase family of enzymes, can be functionally expressed in eukaryotic cells and can promote the deletion of DNA sequences located between directly oriented target sites in mammalian cells. β-Recombinase appears to have high avidity for the nuclear compartment since, following transfection, it was detected mainly in the nuclear region, forming a very condensed and speckled pattern on indirect immunofluorescence. This point was reassessed in subcellular fractionation experiments (see "Results" and Fig. 3). This behavior is similar to that observed for the Cre enzyme (13). Cre and β-recombinase do not present a canonical or bipartite nuclear localization motif in their primary sequence (34, 35). Since they have access to the nuclear compartment, it is assumed that this localization occurs by diffusion through the nuclear membrane or following the transient disorganization of this membrane during mitosis.

Transient β-recombinase expression by plasmid pSVβ2 promoted site-specific recombination between the two directly oriented six sites in the substrate plasmid pCB8 when both plas-
chromatin-associated proteins from plants can also assist recombination (20, 28). It has recently been observed that designing experiments to activate the expression of genes of associated protein can efficiently stimulate indeed, it is known that the mammalian HMG1 chromatin-protein (28), we assume that this factor is provided by the host.

mid recombinase; see Fig. 5). As expected, high expression of expression experiments. This reporter gene was designed to be expressed only upon recombination due to expression was done by A. Varas and M. A. Gallardo. We also thank Coral Bastos and Catherine Mark for secretarial and editorial support, respectively.

Since in vitro recombination requires a chromatin-associated protein (28), we assume that this factor is provided by the host. Indeed, it is known that the mammalian HMG1 chromatin-associated protein can efficiently stimulate in vitro β-mediated recombination (20, 28). It has recently been observed that chromatin-associated proteins from plants can also assist β-recombinase in mediating DNA recombination (29), suggesting that β-recombinase might be also suitable for manipulation of plant genomes.

We have also studied the ability of β-recombinase to act on chromatin-integrated target substrates. Several stable NIH/3T3 clones were established bearing different copy numbers (5–75) of the substrate plasmid pCBS randomly integrated in the host chromatin. Transient β-recombinase expression led to the excision of the sequences between the two directly oriented six sites; the recombination product was detected by PCR amplification from purified genomic DNA and Southern hybridization, and its identity was confirmed by direct DNA sequencing of the amplified product (data not shown).

We provide the first evidence, in eukaryotic cells, for the activity of a DNA recombinase belonging to the prokaryotic resolvase/invertase family. Enzymes of this family promote DNA recombination through a mechanism different from that of DNA integrases. Integrases such as Cre or Flp promote intramolecular as well as intermolecular recombination, whereas recombinases of the resolvase/invertase family are highly specialized in intramolecular recombination. If confirmed in animal models, the availability of a tool such as β-recombinase will expand the possibilities for the programmed modification of eukaryotic genomes currently under use. β-Recombinase, used alone or in combination with the already existing recombination systems, will allow a more specific spatiotemporal control of the recombination events. Researchers would have the opportunity to design several independently controlled recombination events in the same animal or cell, thus providing new, more flexible solutions to general research. In this respect, different approaches to assess whether all these recombination systems can work simultaneously will be of great interest for further investigations.

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