New Insights into Host Factor Requirements for Prokaryotic β-Recombinase-mediated Reactions in Mammalian Cells

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Vicente Díaz‡, Pilar Servetto‡, Ignacio Prieto‡, Manuel A. Gonzalez‡, Carlos Martínez-A.‡, Juan C. Alonso§, and Antonio Bernad¶

From the ‡Departamento de Inmunología y Oncología and ¶Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Cantoblanco, E-28049 Madrid, Spain

The prokaryotic β-recombinase catalyzes site-specific recombination between two directly oriented minimal six sites in mammalian cells, both on episomal and chromatin-integrated substrates. Using a specific recombination activation gene expression system, we report the site-specific recombination activity of an enhanced green fluorescent protein (EGFP) fused version of β-recombinase (β-EGFP). This allows expression of active β-recombinase detectable in vivo and in fixed cells by fluorescence microscopy. In addition, cellular viability is compatible with a substantial level of expression of the β-EGFP protein. Using fluorescence-activated cell sorting, we have been able to enrich cell populations expressing this fusion protein. Application of this strategy has allowed us to study in more depth the host factor requirements for this system. Previous work showed that eukaryotic HMG1 protein was necessary and sufficient to help β-recombinase activity in vitro. The influence of ectopic expression of HMG1 protein in the recombination process has been analyzed, indicating that HMG1 overexpression does not lead to a significant increase in the efficiency of β-recombinase-mediated recombination both on episomal substrates and chromatin-associated targets. In addition, β-recombinase-mediated recombination has been demonstrated in HMG1-deficient cells at the same levels as in wild type cells. These data demonstrate the existence of cellular factors different from HMG-1 that can act as helpers for β-recombinase activity in the eukaryotic environment.

Site-specific recombinases have become a routine tool for conditional gene modifications, as an alternative to classical gene targeting technologies (1–3). These systems, which allow programmed intermolecular recombination, overcome some of the limitations of the classical knock-out systems, such as embryonic lethality or generation of compensation mechanisms. Cre-loxP is currently the system of choice, because of its ability to induce targeted changes in model animals (1, 4). Using this system, tissue-specific, conditional, and inducible gene targeting events in a wide variety of tissues and organs have been reported (5–8). Although the utility of these systems is unquestionable, to date only the Cre-loxP, and to a lesser extent, the Flp/FRT systems are currently under use. Only very recently, the Int/attP site-specific recombination system from λ phage has proven to work successfully in plants (9). Moreover, there are relatively few reports on the combined use of these systems (10). It becomes important to study and characterize other site-specific recombination systems (SSR)1 that could be used as an alternative to or in combination with existing ones.

β-Recombinase from the Gram-positive plasmid pSM19035 induces specific intramolecular recombination in mammalian cells, both in episomal plasmids and in chromatin-associated substrate structures (11). Plasmid pSM19035 has an unusual structure, because almost 80% of the molecule consists of a repeated sequence and two replication origins (12). Because replication of this plasmid follows the classical theta model, a mechanism must exist to ensure its complete replication (inversion) and maximization of plasmid segregation (resolution). The development of an in vitro recombination system based on the use of purified β-recombinase demonstrated both inversion and deletion activities associated to the protein (13), delimited the sequences required for directing the SSR reactions (13, 14), and characterized the requirements for both reactions (13). Unlike Cre and Flp, which belong to the Int recombinase family, β-recombinase is included in the family of resolvases/invertases and has the interesting property of catalyzing exclusively intramolecular recombination events (13, 15). At difference from Cre and Flp site-specific recombinases, which do not require additional factors (reviewed in Refs. 16 and 17), β-recombinase requires for deletions a supercoiled substrate and a chromatin-associated protein (e.g. bacterial Hbsu or eukaryotic HMG1 proteins) (14, 15, 18). We showed previously that the mammalian cell environment can provide such a host factor and that nuclear genomic DNA supercoiling is suitable for β-recombinase function (11).

To obtain additional insights regarding β-recombinase system application for the manipulation of eukaryotic genomes, we have developed a recombination-activated gene expression (RAGE) system dependent on β-recombinase activity that consists of the lacZ reporter gene separated from the promoter by

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1 The abbreviations used are: SSR, site-specific recombination; RAGE, recombination-activated gene expression; GFF, green fluorescent protein; EGFP, enhanced green fluorescent protein; wt, wild type; KO, knock-out; FACS, fluorescence-activated cell sorting; bp, base pairs; PCR, polymerase chain reaction; β-Gal, β-galactosidase; kb, kilobase(s); MOPS, 4-morpholinepropanesulfonic acid.

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the pac gene flanked (sited) by two minimal six sites (19) in direct orientation, equivalent to those described for other models (1–3, 20). In addition, we have also obtained and characterized a novel hybrid protein, composed of β-recombinase fused through its C-terminal end to enhanced green fluorescent protein (EGFP) from Aequorea victoria. As for the Cre-loxP system (21), combination of these two approaches results in a simple, rapid way to enrich cell clones that have undergone the desired recombination event after β-EGFP transient expression, using fluorescence-activated cell sorting (FACS). This approach may be used to increase the efficiency of locus-specific recombination events during the generation of mouse conditional knock-out approaches or for the deletion of the positive selection cassette in mouse conventional knock-out models, which might alter or influence the final phenotype (10, 22).

Using these novel approaches we have studied the host protein factor requirement for β-recombinase protein in mammalian cells. In previous works, several proteins were demonstrated to replace in vitro the chromatin-associated factor required by β-recombinase reaction (15, 18). More precisely, histone-like proteins Hbsu and HU can efficiently contribute to the recombination process. The identity of this host factor in eukaryotic environments is still unknown, although different HMG1-like proteins of mammalian and plant origin have the ability to facilitate the formation of nucleoprotein structures to different extents, because they can efficiently replace a bacterial chromatin-associated protein required for the site-specific β-mediated recombination (15, 18, 23, 24). HMG1/2, which are abundant components of the eukaryotic nucleus, bind any linear DNA with moderate affinity and no sequence specificity (25) but bind with high affinity to DNA that is already sharply bent (26–28); thus they are considered structural chromatin proteins expressed in all eukaryotic cell types. However, recent evidences have demonstrated that HMG1/2 are also recruited to DNA by interactions with proteins required for basal and regulated transcription (29–34) and for SSR machinery, as the RAGV/DJ recombination system (reviewed in Ref. 35), the major determinant for diversity generation of antibody and T-cell receptor. These novel data strongly suggest an additional role as expression regulator of specific gene families or physiological molecular events mediated by the recombination machinery (36). Therefore, HMG1 and other closely related mammalian proteins (i.e. HMG2 and HMG4) are potential candidates to act as host factors in β-recombinase reactions in mammalian cells, although HMG2 is expressed at a much lower level than HMG1 (37), and HMG4 is almost exclusively expressed during embryonic stages of development (38).

Here we show that overexpression of HMG1 does not induce a significant enhancement on β-mediated recombination both on episomal plasmid-based targets or chromatin-integrated constructs. Furthermore, β-mediated recombination is efficiently performed in a HMG1 knock-out cell line. These data clearly suggest that in eukaryotic environments there are other chromatin-associated proteins that could act as helpers in β-mediated reactions, and therefore it is expected that those reactions could occur in most cell types and tissues. In conclusion, the results presented highly support the future usefulness of the β/six system as a new tool for conditional modification of eukaryotic genomes.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Plasmids pBT233 and pCB103 have been previously described (12, 19). Plasmids pEGFP-N1, ppgal-Basic and pPur (CLONTECH, Palo Alto, CA), and pGEM-T (Promega, Madison, WI) were from commercial sources. Plasmid pHMG1 (36) was kindly provided by Dr. M. Bianchi (Milan, Italy).

To fuse the β-recombinase gene to EGFP in the pEGFP-N1 vector, we introduced appropriate 5′ HindIII and 3′ EcoRI sites on the β gene from pBT233. Using primers βHind5 (5′-GAGAAGAGCTTGGTTGAAAATGGCT-3′) and βEco3C (5′-ACTATCCCTCTTCCTCC-3′), we performed PCR amplification (30 cycles at 94 °C 1 min, 48 °C 1 min, 72 °C 1 min, followed by one cycle of elongation at 72 °C 5 min). The 635-bp amplification product was joined to HindIII/EcoRI-digested pEGFP-N1 followed by standard cloning procedures. The resulting plasmid pβ-EGFP was purified for transfection experiments with Qiagen columns.

For recombination-activated gene expression, we designed a set of three vectors that were obtained as follows. A 93-bp site was obtained by PCR amplification from pCB103, for which we used the pUC reverse primer and a primer containing a novel Smal1XmaI site needed for following which a 93-bp fragment called 103M (5′-CATGGCCAAGCTTCCGGGGCTGCAGG-3′). The amplified product was purified and cloned using the pGEM-T Easy system, giving rise to the pGEM-T/103M plasmid. This was digested with KpnI and HindIII, and the resulting fragment containing the six site was cloned in the same restriction sites of the ppgal-Basic vector, resulting in the psgixgal vector containing the lacZ gene downstream of a six site. The BamHI fragment containing the six-loxZ cassette from this vector was cloned in pPur vector (CLONTECH), resulting in the pPursigal plasmid, the negative control vector used in all experiments (see Fig. 2A). The last six site was obtained from psgal by digestion with XmaI and cloned in the AgeI site from pPursigal to obtain Recombiner, the substrate for recombination used in this study (see Fig. 2A). The pgal vector (positive control) was used in vitro recombination essentially as described (12, 13), using 1 μg of Recombiner as substrate, 320 nm purified β-recombinase, and 100 nm Hbsu. The resulting reaction was digested with SaciI, which only cuts in Recombiner molecules but not in pgal. Transformation of this restriction reaction rendered pgal containing bacterial colonies for further purification of this plasmid.

For retroviral transduction of the HMG1-defective cell line, the β-EGFP open reading frame from pβ-EGFP plasmid was cloned in pLZR-CMV-GFP retroviral vector (39) upon digestion with BamHI and NotI restriction enzymes and subsequent ligation. The resulting plasmid, pLZR-β-EGFP, was further packaged in retroviral particles as described below.

**Cell Lines and Cultures**—NIH/3T3 cell line was obtained from the American Type Culture Collection (CRL-1658). 293T cells were originally called 293tsA1609ts (40) and derived from 293 cells, which are a human embryonic kidney cell line. HMG1 KO and wt counterpart cell lines were a gift from Dr. M. Bianchi (36). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Cultek, Madrid, Spain) and 2 mM l-glutamine (Merck).

**Transfection and Retroviral Transduction**—To obtain stable transformants of the β-EGFP fusion or the recombination activable Recombiner construct, plasmid DNA (20 μg) was introduced in NIH/3T3 cells by electroporation of 2 × 106 cells at a concentration of 106 cells/ml in supplemented Dulbecco’s modified Eagle’s medium, pulsed at 220 V, 950 microfarads (Bio-Rad Gene Pulser). Cells were replated, and after 48 h of further culture, selection antibiotics were added to the medium. Transfection of stable cell clones bearing the β-EGFP plasmid. For this we used 1 mg/ml G418, and for the Recombiner construct, 2 μg/ml puromycin. Clones were collected and analyzed 10–15 days after electroporation. Transient transfection was done in all cases using Superfect reagent (Qiagen), according to manufacturer’s instructions. FACS analysis and β-Gal detection were performed 48 h after transfection.

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**Fluorescence Microscopy and FACS**—In vivo expression of β-EGFP fusion was easily detected by direct observation of cultures in a phase-contrast microscope equipped with a fluorescent lamp and blue filter. Indirect immunostaining was performed after fixing cells with 3% formaldehyde, 5% Triton X-100 for 20 min at room temperature. Blocking was performed with 100 mM NH4Cl in phosphate-buffered saline for 10 min. A rabbit-β-recombinase rabbit polyclonal antibody (1) was used at a 1:500 dilution. Secondary Cy3-labeled goat anti-rabbit IgG (H + L) antibody was purchased from Amersham Pharmacia Biotech and used at 1:400 dilution. For Hoechst 33258 staining, cells were incubated with 0.8 μg/ml Hoechst 33258 (Molecular Probes, Eugene, OR) for 5 min, immediately after second antibody incubation. Samples were mounted and analyzed using a Leitz DMINB epifluorescence microscope and a Leica...
RESULTS

β-EGFP Fusion Behaves as a Nuclear Protein That Can Be Constitutively Expressed in Mammalian Cells—The β-recombinase open reading frame was cloned into the pEGFP-N1 vector to fuse this gene with the 5' end of the EGFP gene. The resulting pβ-EGFP plasmid was transiently transfected in NIH/3T3 cells. In contrast to transfections with pEGFP-N1, fluorescence microscopy showed a nucleus-restricted pattern in the transfected cells (Fig. 1A). This nuclear signal was observed more clearly using confocal microscopy (Fig. 1B). The nuclear pattern is not homogeneous, because β-EGFP recombinase localizes in heterochromatin regions. This compartmentalization was confirmed by counterstaining other gene products that localize in these regions (not shown). Furthermore, living mitotic cells expressing the fusion protein show brighter signals in the centromeric areas of the chromosomes (Fig. 1B), which have been assigned to heterochromatin (42). The β-EGFP signal was confirmed by immunostaining with anti-β-recombinase specific antibodies (not shown). These results confirm and extend our previous observation on the spontaneous subcellular localization of β-recombinase in eukaryotic cells (11).

To assess the feasibility of stable β-EGFP fusion protein expression in living cells, plasmid pβ-EGFP was transfected in NIH/3T3 cells, and stable clones were obtained. Different levels of expression of β-EGFP were observed when analyzed by fluorescence microscopy (Fig. 1C, clones B3, D3, and D4) and by cytometry (Fig. 1C, lane 3). We were unable to obtain clones with very high expression levels, suggesting a deleterious effect of either high doses of β-recombinase or EGFP expression, although results in other systems argue for an EGFP-mediated toxic effect (43). Selected β-EGFP-expressing clones developed normally (Fig. 1C, lane 1), and growth rate parameters were equivalent to those of wt cells (not shown), demonstrating that sustained β-EGFP expression is compatible with normal cell processes. The NIH/3T3 clone β/EGFP.D4 was selected for further experiments because of its high, stable, and uniform β-EGFP expression pattern.

The β-EGFP Protein Catalyzes SSR—To elucidate whether fusion to EGFP would alter the enzymatic activity of β-recombinase, β/EGFP.D4 cell clone was transiently transfected with the Recombiner construct (11) containing the minimal directly oriented six sequences (93 bp), which direct β-recombinase-mediated deletions (19). The Recombiner reporter system was designed for in vitro expression of β-Gal activity after β-recombinase-mediated elimination of the mixed pur open reading frame (Fig. 2A). The structure of the Recombiner reporter system was tested in vitro as a suitable substrate for β-recombinase activity (Fig. 2B), and the control reporter plasmid pgal was isolated from the in vitro reaction (Fig. 2B, lane C2). The insertion of six sites upstream of or flanking the reporter genes used in this RAGE system does not appear to interfere with normal gene expression (Fig. 2C). This is important in genomic tissue-specific modification experiments, in which it is essential to maintain the original expression levels of the gene to be deleted before conditional manipulation, and in tissues other than that designed to express β-recombinase. Conversely, constitutive expression through the recombinase substrate does not appear to affect β-recombinase function (Fig. 2D).

After transfection with Recombiner or with control plasmids, pPursigal and pgal, cell protein extracts were obtained for quantitative analysis of β-Gal expression. β-Gal activity was detected only in transfections with Recombiner and pgal constructs (Fig. 2D), but not in the negative control and pPursigal, which is not a suitable β-recombinase substrate because it amounts of each extract were analyzed. Luminescence was measured in a scintillation counter (Wallac, EG&G Instruments, Madrid, Spain).

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lacks one of the two directly oriented six sites required for the recombination process. Taken together, these results demonstrate that β-EGFP protein maintains the recombinase activity and suggest that fusion of other protein domains at the β-recombinase C terminus would not affect its activity.

Combined Use of RAGE and β-EGFP Fusion for the Improved Selection of Site-specific Genome-modified Cellular Clones—The use of β-EGFP fusion in combination with the developed RAGE system has been also useful for isolation of cellular clones harboring site-specific recombined chromatin-associated targets. The Recombiner plasmid was transfected in the NIH 3T3 cell line, and several resistant clones were obtained after puromycin selection. Clone 3T3/D6.3 was selected for further study, because it demonstrated the highest β-Gal levels after β-EGFP expression (not shown). This clone harbored several copies of the target sequence (not shown). In a first approach, we generated resistant clones after transfection of the 3T3/D6.3 line with pβ-EGFP or pEGFP-N1 plasmids, followed by G418 selection. Clones from the pβ-EGFP transfection (Fig. 3A, lanes I–6) and from pEGFP-N1 (lane C) were recultured and analyzed. Both the Southern analysis and β-Gal detection showed heterogeneous recombination levels in the different clones when compared with the untransfected clone (Fig. 3A, lane C). These results indicate the feasibility of the approach and suggest a distinct accessibility of β-recombinase to the copies of Recombiner structure within the chromatin of clone 3T3/D6.3, probably dependent on the distinct level of the β-recombinase induced in the cell clones.

In an experimental design similar to that previously described with the Cre-loxP system (21), clone 3T3/D6.3 was transiently transfected with the pβ-EGFP plasmid; 48 h later, cells were trypsinized and analyzed by flow cytometry. The typical fluorescence distribution in these cells after transfection with pβ-EGFP or pEGFP-N1, rendered 12–20% positive cells (not shown). We sorted populations of moderate-high expression (+, levels similar to those obtained in stable clones; see Fig. 2A), very high expression (++), and no or very poor expression (−). Sorted cells (85–95% enriched) were further cultured in growth medium without antibiotic selection until isolated colonies had formed, and each one was processed separately for genomic DNA isolation and PCR analysis (see Fig. 2A for scheme). The PCR amplified fragments were separated on agarose gels, blotted, and hybridized with a probe for the lacZ gene to ensure detection and specificity of the amplified diagnostic product (0.4 kb). Almost 80% of the + group (intermediate-high expression) showed signal amplification (Fig. 3B), whereas in the ++ group (very high expression), only 50% were recombination-positive clones. This result again suggests some type of deleterious effect in the ++ group, concuring with the expression levels observed in stable plasmid pβ-EGFP clones.

HMG1 Is Not an Essential Requirement for the β-Recombinase-mediated Reactions in Mammalian Environments—The need for a host factor to ensure in vitro recombination in the β/six system has been previously reported (13, 15). The host factor is also provided in eukaryotic environments, but its...
identity remains unknown. In vitro reactions with HMG1 proteins of both plant and animal origins have proven to work as efficient accessory factors for β-mediated recombination (18, 23). HMG1 and other members of the same family are thus good candidates for host factors in β-mediated recombination reactions in eukaryotic cells. We tested the influence of ectopic HMG1 expression in in vivo reactions carried out with Recombiner as substrate. Transient cotransfections with a HMG1 expression plasmid (36) and Recombiner in βGFP.D4 cell line, resulted in a positive influence on the β-mediated recombination on the episomal substrate when detected by RAGE (Fig. 4A). Overexpression of HMG1 in the transfection experiments was confirmed by semiquantitative PCR (Fig. 4C) using specific primers for the corresponding mRNA (see “Experimental Procedures”). It has been previously described that ectopic HMG1 expression could promote an enhancer effect on expression levels through different promoters (34). To evaluate whether this effect could be mediating in the increment observed in β-Gal expression, we performed transient pHMG1 and/or pgal transfections in the β/GFP.D4 line (not shown), demonstrating such an effect (approximately 2-fold) on SV40-directed β-Gal expression. This increment seems to account for most of the total effect obtained in the RAGE recombination analysis (Fig. 4A). This conclusion has been confirmed with independent criteria, making use of quantitative PCR for the monitoring of the recombination events (Fig. 4D). In this set of experiments we were not able to demonstrate any reproducible enhancement of the β-mediated recombination of the Recombiner substrate when HMG1 was also cotransfected in the cells. We therefore conclude that the enhancement of RAGE β-Gal expression observed (Fig. 4A) is the consequence mainly of the increment in SV40 promoter activity and not of a significant improvement in the recombination rate.

The influence of HMG1 in chromatin-associated targets was also evaluated. Fig. 4B shows the result of the transfection of β-EGFP alone or combined with pHMG1 in the 3T3/D6.3 line. In this case, overexpression of HMG1, also monitored by semiquantitative PCR (not shown) did not show a remarkable effect. In addition, we also confirmed that overexpression of HMG1 by cotransfection with the β-GFP clone does not appear to influence the efficiency of the β-mediated recombination nor the distribution of β-recombined clones in 3T3/D6.3 line, after sorting/cloning experiments equivalent to those previously described (Fig. 3B). Again, the recombined clones are detected exclusively within the intermediate positive clones with a mean efficiency of the 70%, remarking some type of toxic or deleterious effect because of high β-EGFP expression levels.

Finally, to address whether HMG1 is a critical factor for the β-mediated recombination processes, we analyzed the efficiency of the specific β/six system recombination in a HGM1-defective skin fibroblast murine cell line, derived from the knock-out model (36). For that purpose we developed a retroviral vector for the stable transduction and expression of the β-EGFP protein in this HMG1-knock-out cell line in comparison with a control one, derived from wt littermates. Transduced and GFP-sorted HMG1 knock-out and wt cell populations were further transfected with the reporter Recombiner plasmid. Analysis by semiquantitative PCR of the recombination products generated during the first 24 h after the transfection of Recombiner in β-EGFP-transduced HMG1-defective cells showed that β-mediated recombination occurs, in absence of HMG1, at a very comparable level with respect to the HMG1 wt control cell line (Fig. 5), indicating the existence of other eukaryotic cellular factors that can act as helpers of the recom-
catalyze resolution reactions (18). In a previous report, we showed that β-recombinase is active in eukaryotic cells (11), implying the existence of at least one eukaryotic host factor that can contribute to the recombination reaction and that the eukaryotic chromatin organization can provide the supercoiling needed for β-mediated recombination.

To evaluate in more depth β-recombinase activity in the eukaryotic environments, we developed an easily detectable fusion protein that maintains both the recombinase activity and the subcellular localization properties described for this protein (Ref. 11 and Figs. 1 and 2). The data presented here clearly demonstrate that the β-EGFP fusion presents no major modifications in its recombination activity. It is therefore possible to assume that the C-terminal region of β-recombinase is flexible and that fusion of other protein regulatory domains would behave similarly. This result opens the future possibility of introducing regulatory domains for temporal-inducible control of β-recombinase activity, as the fusion of the ligand-binding domain from receptors corresponding to several hormone systems, as shown for the Cre-loxP model (48–50).

The use of β-EGFP protein also revealed a stronger preference of β-recombinase for the heterochromatin regions within the nucleus. The preferential nuclear localization can be explained by the bipartite nuclear localization signal (51–53) present in the C-terminal portion (amino acids 184–200) of β-recombinase, potentially implicated in active transport to the cell nucleus via specific mechanisms in eukaryotic cells. We also show that sustained β-recombinase activity is compatible with normal cell growth (Fig. 1). Stable β-EGFP expressing clones were developed after pβ-EGFP transfection of NIH/3T3 cells, and they show homogeneous expression of the chimeric protein, which is easily detected both by in vivo microscopy and by flow cytometry.

The existence of recombination in a transcriptionally active structure (Recombiner) ensures that the transcription machinery does not interfere with the recombination process. Conversely, the insertion of six sites between the promoter regions and the gene to be expressed has no significant effect on the transcription level (data not shown). This is an important feature for a system intended for use in conditional gene manipulation, because the goal of such an approach is to maintain unmodified levels of the gene product of interest (designed as a “floxed/sixed” construct) before induction of the targeted deletion, or outside the tissue/organ in which recombination is expected to occur. The development of the described RAGE system coupled to the use of the β-EGFP version of the β-recombinase has allowed the efficient enrichment of populations expressing β-recombinase activity and the evaluation of several mechanistic and practical aspects of the β/six system applied to the targeted modification of the mammalian genome.

The role of HMG1 as a putative critical host factor in β-mediated recombination was first evaluated through the analysis of the consequences of HMG1 overexpression on the β-mediated recombination efficiency. Cotransfection of a plasmid encoding HMG1 in experiments parallel to those described above seemed to show a clear enhancing effect on recombination efficiency (Fig. 4A). When Recombiner is introduced as an episomal substrate, HMG1 overexpression promotes a 3-fold increment in β-Gal expression compared with transfections with Recombiner alone. However, previous reports showed that HMG1 expression enhances the expression of promoters such as SV40 early promoter or cytomegalovirus (34). By analyzing the effect of HMG-1 overexpression on the levels of β-galactosidase expression in the recombinant product and measuring recombination efficiency by using quantitative PCR, we determined that the observed effect was mainly due to this recom-

**Fig. 3.** A, β-EGFP-mediated recombination on chromatin-associated targets. Clone 3T3/D6.3 was transfected with plasmid pβ-EGFP, and several double-stable clones were analyzed. Protein extracts of these clones were prepared, and β-Gal activity was measured as described under “Experimental Procedures.” The upper panel graphs the β-Gal activity for each clone; the lower panel shows a Southern analysis of genomic DNA from these clones digested with EcoRV and SacII and hybridized with a lacZ gene-specific probe. The positions of bands of interest are indicated. Lane C, untransformed clone. Lanes 1–6, different stably transfected clones. Intensity ratios (1.9-kb band/1.4-kb band) for each lane: 1.8, 2.0, 1.0, 0.46, 1.74, and 1.58. B, recombination-activated gene expression of cells expressing β-EGFP fusion protein after cell sorting. Genomic DNA of several individual clones was prepared and analyzed by PCR using sixUP and sixDO primers to detect recombination events. The resulting products were separated on agarose gels amplification and blotted. The upper panel shows the result of hybridization with a lacZ-specific probe. The positive control of this PCR reaction (+) was prepared using 100 ng of plasmid pgal as template. To ensure homogeneous loading of genomic DNA in each sample, a control PCR reaction was performed to detect the TSHβ gene (0.4-kb fragment, lower panel). In this case, the positive control consisted of 500 ng of mouse genomic DNA. In both PCR reactions, the negative control (−) corresponds to a mock reaction amplification without template DNA.

**DISCUSSION**

SSR technologies have widely expanded the possibilities of genomic manipulation in living organisms. With these systems, strict spatio-temporal control of the induced manipulation can be achieved, eliminating the consequences of undesired lethal effects because of systemic lack of an essential gene (see Refs. 44 and 45) or bypass effects because of uncontrollable redundant mechanisms that result in the absence of detectable phenotype changes (46, 47).

In contrast to Cre-loxP and Flp/FRT systems, β-six model requires a host protein factor and a supercoiled substrate to
Fig. 4. Influence of HMG1 expression in β-mediated recombination in the βGFP.D4 clone (A) and in the 3T3/D6.3 clone (B) of the indicated plasmids. At 48 h after transfection, proteins were extracted, and β-Gal activity was measured. Each transfection was performed in triplicate; the representations correspond to the mean value obtained from each triplicate condition ± S.D. expressed in relative units (RU). 1 unit is arbitrarily assigned to β-Gal activity detected in pUC18 transfection (background levels). C, upper panel, detection of the expression of HMG1 mRNA upon transfection with the indicated plasmids. Total RNA from transfected cells was extracted 48 h after transfection, and the cDNAs corresponding to mRNAs was obtained using RT. HMG1 cDNA levels were estimated using semi-quantitative PCR conditions. The number of cycles was adjusted to nonsaturation conditions. The lower panel corresponds to the detection of the housekeeping gene GAPDH. HMG1 KO cell line was used as negative control for this detection. D, quantification of recombination levels upon Recombiner co-transfection with (+ pHMG1 lane) or without pHMG1 plasmid (− pHMG1 lane) in βGFP.D4 cell line using quantitative PCR (see “Experimental Procedures”).

Fig. 5. β-Mediated SSR in HMG1-deficient cells. Upper rows, detection of recombination events by PCR using specific primers (see “Experimental Procedures”) in HMG1 wt and HMG1 KO cell lines. – lane, Recombiner plasmid. + lane, pgal plasmid. HMG1 KO cell line and the HMG1 wt control cell line were transduced with the pLZR-β-EGFP retroviral vector for constitutive and stable expression of β-GFP fusion protein. After sorting and reculture of the GFP positive cells, these were transfected with the indicated plasmids and the levels of recombination were evaluated by semi-quantitative PCR in the indicated conditions (see “Experimental Procedures”). β-GFP transduced lanes correspond to transfections in HMG1 wt or KO cells retrovirally transduced with pLZR-β-EGFP retroviral vector (see “Experimental Procedures”). The lower rows show PCR detection of LacZ gene, which was used as an internal loading control for the reaction.

...combination-independent effect (Fig. 4D), although we could not rule out completely a slight enhancement on the recombination structure. HMGs may stabilize the recombination complex by providing minor groove DNA contacts in the vicinity of the specific recombination sequences in this system (35). However, this mechanistic implication was not formally substantiated from the results obtained recently on the HMG1 knock-out model (36), where no great defect was noted in the immune repertoire.

In the β-mediated six-specific recombination system, the exogenous introduction of HMG1 expression constructs does not exert a remarkable effect, neither on episomal target plasmids nor in chromatin-associated target structures, suggesting that HMG1 is not a critical factor for the β-mediated recombination in eukaryotic environments. To confirm this suggestion, we attempted to define whether HMG1 was the principal contributor to β-recombinase-mediated reaction in eukaryotic cells. We performed transfections of Recombiner in a HMG1-defective cell line expressing β-EGFP fusion and searched for recombination products. PCR detection showed the existence of efficient recombination caused by β-recombinase in this HMG1-defective environment (Fig. 5). These data indicate that there are other cellular factors that, at least, can substitute for HMG1 in the recombination reaction. Moreover, episomal targets could adopt in the mammalian nucleus a recombination-efficient structure caused by organization of a chromatin-like structure in such environment, regardless of specific factors. This would minimize possible problems derived from a theoretical unavailability of factor in experimental applications of the β-recombinase system.
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