Transcriptional Repression Mediated by Polycomb Group Proteins and Other Chromatin-associated Repressors Is Selectively Blocked by Insulators*

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Polycomb group (PcG) proteins repress gene activity over a considerable distance, possibly by spreading along the chromatin fiber. Insulators or boundary elements, genetic elements within the chromatin, may serve to terminate the repressing action of PcG proteins. We studied the ability of insulators to block the action of chromatin-associated repressors such as PcG proteins, HP1, and MeCP2. We found that the Drosophila special chromatin structure insulator completely blocks transcriptional repression mediated by all of the repressors we tested. The Drosophila gypsy insulator was able to block the repression mediated by the PcG proteins Su(z)2 and RING1, as well as mHP1, but not the repression mediated by MeCP2 and the PcG protein HPC2. The 5′-located DNase I-hypersensitive site in the chicken β-globin locus displayed a limited ability to block repression, and a matrix or scaffold attachment region element was entirely unable to block repression mediated by any repressor tested. Our results indicate that insulators can block repression mediated by PcG proteins and other chromatin-associated repressors, but with a high level of selectivity. This high degree of specificity may provide a useful assay to define and characterize distinct classes of insulators.

Insulators have been identified in various species. The Drosophila scs and scs′ (special chromatin structure) boundary elements have been found to flank the hsp70 heat shock locus in Drosophila (6–8). The Drosophila gypsy insulator (4, 9, 10, 11) contains a cluster of 12 binding sites for the Su(Hw) (suppressor of hairy wing) protein (4, 11). A vertebrate boundary element is the 5′-located DNase I-hypersensitive site (5′-HS) in the chicken β-globin locus (12–14). Finally, matrix or scaffold attachment regions (MARs/SARs) have been postulated to function as insulators (15–17).

Two assays are commonly used to test whether a DNA sequence is a functional insulator. In the enhancer blocking assay, a putative insulator is cloned between an enhancer and a promoter. The scs/scs′ and gypsy insulators as well as the 5′-HS element are able to efficiently prevent enhancer-promoter interactions (6, 7, 12, 18). In another, frequently used assay, it is tested whether a putative insulator confers position-independent expression on an eye color (white) reporter gene in Drosophila. Stable integration of the white gene into the genome normally results in variable expression of the white gene, as monitored by the eye color. This variability is due to the random integration of the transgene into the genome. When the white gene is flanked by the scs and scs′ insulators, the expression of white becomes less variable between transformants (6). This has been interpreted as an indication that the insulators shield the transgene from either activating or repressing effects, emerging from the surrounding chromatin. This assay is, however, much less defined than the enhancer-blocking assay. In the enhancer-blocking assay, specific DNA sequences cloned in a defined construct can be tested in a controlled assay system. The position dependence assay, on the other hand, relies on random integration of a transgene into various positions in the genome and is, therefore, less defined. To study the ability of insulators to block transcriptional repression in a more controlled manner, we developed a repression assay in a human cell line, to monitor repression of a reporter gene by specific, well defined repressors. We tested whether insulators protect against repression, mediated by the PcG proteins HPC2 (19), RING1 (20, 21), and Su(z)2 (suppressor of gypsy) (22, 23). We compared these proteins with two other, chromatin-associated repressors, the mouse homolog of heterochromatin protein 1, mHP1 (24–26), and the methyl-CpG-binding protein, MeCP2 (27, 28). We found that the various insulators are able to block repression that is mediated by chromatin-associated repressors but with a high level of selectivity. The scs insulator was most effective in blocking repression, whereas the MAR/SAR element was completely ineffec-

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* The abbreviations used are: PcG, polycomb group; scs, special chromatin structure; 5′-HS, 5′-located DNase I-hypersensitive site; MAR/SAR, matrix or scaffold attachment region; bp, base pair(s); kbp, kilobase pair(s); Tet, tetracycline; HSF, heat shock factor.
of six 5'-HS core elements (13), or a 1.0-kbp fragment encompassing the 5'-globin locus (12), the 1.7-kbp-long array of six 5'-HS core elements (13), or a subsequent fragment encompassing the Drosophila histone MAR/SAR element (16, 29).

The Inducible Tet-off System—We established the Tet-off system for induction of the LexA repressor fusions in the human U-2 OS osteosarcoma cell line. The pTET-off vector (CLONTECH) encoding a fusion protein between the Tet repressor and the transactivator VP16 was stably transfected under Geneticin (G418) selection pressure. Subsequently, the LexA repressor fusion cDNAs were stably transfected into U-2 OS cells containing the respective reporter constructs were grown to ~90% confluence. Cells were harvested as described above, with the following exception. The cells were resuspended in 200 μl of reaction buffer B (150 mM sucrose, 80 mM KCl, 35 mM Heps, pH 7.4, 5 mM K₃[HPO₄]₅, 5 mM MgCl₂, 2 mM CaCl₂). Approximately 10⁶ cells in 100 μl of reaction buffer B were digested by adding 100 μl of reaction buffer B containing 0.4% Nonidet P-40 and different amounts of DNase I (Roche Molecular Biochemicals; grade I). Reaction conditions and DNA purification were identical to those described above. Subsequently, 4 μg of DNA was cut with EcoRV and EcoNI (Fig. 4), separated on a 1.5% agarose gel, and subjected to Southern transfer. As a probe, we used a [α-³²P]dATP-labeled DNA probe, and the blot was autoradiographed with an intensifying screen at ~70 °C using an x-ray film.

To determine the DNase I sensitivity of the region between the LexA operators and the HSF-inducible promoter, cells were grown in doxycyclin-free medium to induce the LexA, LexA-HPC2, and LexA-mHP1 proteins. After that, cells were harvested and treated with DNase I as described above. Subsequently, 4 μg of the DNA was cut with EcoRV, separated on a 1% agarose gel, and subjected to Southern transfer. We used a [α-³²P]dATP-labeled probe that covers the 1 kbp of a DNA between the LexA operators and the HSF-inducible promoter. The blot was autoradiographed with an intensifying screen at ~70 °C using an x-ray film.

Western Blot Analysis—U-2 OS cells that were stably transfected with the pTRE repressor constructs and transfected with the reporter constructs were lysed prior to the delivery of a heat shock to activate the reporter gene. A portion of the lysate was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The blots were probed with a 1: 5000 dilution of a polyclonal rabbit antibody, directed against LexA (Invitrogen).

RESULTS

An Assay to Monitor Repression of a Reporter Gene in a Human Cell Line—We developed an assay to monitor repression of a reporter gene by chromatin-associated repressors in a controlled fashion. Chromatin-associated repressors such as the PcG proteins and HP1 do not bind directly to the DNA. However, when targeted to a reporter gene as fusion proteins, they act as repressors (23). We concentrated on the following chromatin-associated repressors: the human PcG protein HPC2 (19), the Drosophila PcG protein Su(z)2 (22), the human PcG-associated protein RING1 (20, 21, 30), M51 or mHP1, a murine homolog of the Drosophila heterochromatin protein HP1 (25, 26), and the methyl-CpG-binding protein, MeCP2 (27, 28). These proteins were targeted as LexA fusion proteins to LexA operators, located upstream of a reporter gene. We and others have previously found that when targeted to a promoter these fusion proteins are efficient repressors of gene activity in a variety of cell lines, including COS and BALB/c 3T3 cells (19, 20, 23, 28, 30, 31).

In order to be able to manipulate the levels of the LexA repressor proteins, we established cell lines in which the expression of the individual LexA repressor proteins was induced under control of the Tet-off induction system (CLONTECH).
Fig. 1. Expression levels of induced LexA repressor proteins. Expression of indicated LexA repressors in U-2 OS cells was induced by the removal of doxycyclin from the culture medium. The repressor cDNAs were cloned in fusion with the cDNA encoding the DNA binding domain of the LexA protein (amino acids 1–202). After 48 h, the cells were harvested and lysed. Lysates from cells grown in the presence (−) or absence (+) of 10 ng/ml doxycyclin were tested for the presence of LexA fusion protein by probing the Western blot with an antibody against LexA. The expression of LexA (lanes 1 and 2), LexA-HPC2 (lanes 3 and 4), LexA-RING1 (lanes 5 and 6), Lex-A-Su(z)2 (lanes 7 and 8), LexA-MeCP2 (lanes 9 and 10), and LexA-mHP1 (M31) (lanes 11 and 12) was monitored. Note the absence of LexA repressor proteins under noninducible conditions. Molecular masses (in kilodaltons) are indicated on the left.

We chose the human osteosarcoma cell line U-2 OS to develop this system, since we have previously found that targeted PcG proteins are able to efficiently repress reporter genes in these cells (19, 20). We found efficient induction of the LexA (Fig. 1, lanes 1 and 2), LexA-HPC2 (lanes 3 and 4), LexA-RING1 (lanes 5 and 6), Lex-A-Su(z)2 (lanes 7 and 8), LexA-MeCP2 (lanes 9 and 10), and LexA-mHP1 (lanes 11 and 12) proteins, 48 h after removal of the doxycyclin in the culture medium. Importantly, in the presence of doxycyclin we found no detectable expression of the LexA repressors. In these stable cell lines, we transfected the reporter constructs and monitored the degree of repression, mediated by the LexA repressor proteins.

The LexA repressor proteins were targeted to LexA operators that were cloned upstream of the HSF-inducible promoter (see top of Fig. 3). All reporter constructs were cloned in the Epstein-Barr virus-derived pIREP4 vector (Invitrogen). When placed under hygromycin selection pressure, these vectors do not integrate stably in the genome but instead propagate as episomes. To establish whether in such a system the vectors obtain a bona fide chromatin structure, we tested the nucleosomal chromatin structure of two reporter constructs. The gypsy insulator (Fig. 2a, lanes 1 and 3) and the MAR/SAR element (Fig. 2a, lanes 2 and 4) were cloned between the LexA operators and the heat shock-inducible promoter. We transfected U-2 OS cells that stably express the LexA-HPC2 protein with these reporter constructs. After 24 h, the medium was changed and replaced with medium containing doxycyclin to prevent induction of the LexA-HPC2 protein (Fig. 1). After another 48 h, we treated the cells with micrococcal nuclease. This is the time period after which in our test system normally a heat shock is delivered to activate the heat shock-inducible promoter. Isolated DNA was Southern blotted and probed with the luciferase reporter gene. A nucleosomal ladder was observed (Fig. 2a, lanes 1 and 2), indicating that, 72 h after transfection, the vectors had indeed obtained a nucleosomal chromatin structure. As a positive control, we compared the nucleosomal chromatin structure of the two reporter constructs that had been stably transfected (Fig. 2a, lanes 3 and 4). No significant differences in the nucleosomal ladder were observed between the reporter genes 72 h after transfection (Fig. 2a, lanes 1 and 2) and in the stably transfected reporter genes (Fig. 2a, lane 3 and 4).

The gypsy insulator (lanes 1 and 3) or the Drosophila MAR/SAR element (lanes 2 and 4) was cloned between the LexA operators and the heat shock-inducible promoter. Cells that had either been transfected 72 h before testing (lanes 1 and 2) or that had been stably transfected (lanes 3 and 4) were treated with micrococcal nuclease. Isolated DNA was Southern blotted and probed with the luciferase gene. A nucleosomal ladder was observed in all cases. b, HPC2 and mHP1 induce a chromatin structure that is less sensitive for DNase I. Cells expressing LexA (lanes 1 and 2), LexA-HPC2 (lanes 3 and 4), or LexA-mHP1 (lanes 5 and 6) were treated with increasing amounts of DNase I. Isolated DNA was Southern blotted and probed with a region between the LexA operators and the heat shock-inducible promoter.

We also tested whether, in this system, the chromatin-associated repressors are able to induce transcriptionally inacces- sible chromatin. We incubated cells with DNase I, the isolated DNA was Southern blotted, and we monitored DNase I sensitivity using a probe that covers the 1 kbp of λ DNA between the LexA operators and the HSF-inducible promoter. We observed a decrease in general DNase I sensitivity when either LexA-HPC2 (Fig. 2b, lanes 3 and 4) or LexA-mHP1 was expressed (Fig. 2b, lanes 5 and 6), as compared with expression of LexA alone (Fig. 2b, lanes 1 and 2). This result indicates that, in this system, HPC2 and HP1 are able to induce a chromatin structure that is less sensitive for DNase I.

In summary, we have developed a repression test assay in which inducible LexA repressors are targeted to a reporter gene that is cloned in Epstein-Barr virus-derived vectors. These vectors display hallmarks of proper chromatin structure. Chromatin-associated Repressors Are Able to Repress Gene Activity over a Distance—The LexA repressor proteins were targeted to the LexA operators that were cloned 50 bp, 1 kbp, 2 kbp, or 6.7 kbp upstream of the HSF-inducible promoter (see reporter constructs at top of Fig. 3). This experiment was a necessary first step, since in the experiments that will be described below we test the influence of insulators that are placed between the LexA operators and the promoter. These insulators can be up to 1.7 kbp long (see, for instance, the scs element; Fig. 5). Loss of repression due to the presence of an insulator should not be the result of the inability of a repressor to repress over this long distance. We therefore tested whether the LexA repressor proteins could repress the reporter gene over distance.

We found that all LexA repressor proteins were able to efficiently repress the activity of the reporter gene when the LexA operators were placed immediately upstream of the heat shock-inducible promoter (Fig. 3). All repressors were able to repress the activity of the heat shock-inducible promoter to as low as 15–20% of the control activity. Also, all LexA repressor
proteins were able to repress the activity of the reporter gene when the LexA operators were placed up to 2 kbp upstream of the heat shock-inducible promoter. Over a distance of 2 kbp, the MeCP2 protein was the most efficient repressor (up to 85%; Fig. 3). However, none of the repressors was able to cover a distance of 6.7 kbp of λ DNA between the LexA operators and the HSF-inducible promoter. 24 h after transfection, the cells were washed, and medium with or without doxycyclin was added to repress or induce, respectively, the LexA repressor proteins. After another 48 h, the cells were exposed to a heat shock to activate the LUC reporter gene. LUC activity was normalized to β-galactosidase activity. The LUC activity in cells in which no LexA repressor protein was induced (in the presence of doxycyclin) was set at 100%. LUC activities in cells in which the LexA repressor proteins were induced (in the absence of doxycyclin) were expressed as a percentage of this control. Values are the mean ± S.E. of four independent experiments.

These results indicate that repression in this system can cover a distance of 2 kbp but not of 6.7 kbp. This last characteristic rules out the possibility that repression of the promoter is the result of spreading of repression from the LexA operators. To test whether known insulators are able to block repression mediated by chromatin-associated repressors, we placed the 1.7-kbp scs insulator (6) in both orientations between the LexA operators and the heat shock-inducible promoter (top of Fig. 5). We found that in the presence of the scs element, none of the tested chimeric LexA repressor proteins was able to repress the reporter gene (Fig. 5). This indicates that the scs element is able to efficiently block the repression of gene activity mediated by these chromatin-associated repressors. No difference was observed whether the scs element was either cloned in the 5' to 3' orientation or in the 3' to 5' orientation. The result indicates that the positive effect of the scs element on transcription is due to blocking of repression, which originates from the LexA operators. To rule out the possibility that the scs element might interfere otherwise with transcription, we cloned the scs element upstream of the LexA operators (top of Fig. 5). In this experiment, all repressors were able to efficiently repress the reporter gene (Fig. 5), indicating that the effect of scs on transcription is indeed due to blocking of repression. Another possibility is that the scs element enhances transcription in our assay and that the lack of repression we observe is simply due to compensation by enhanced transcription. However, the scs element in the reporter construct did not induce significant changes in luciferase activity when either no LexA repressors or only the LexA protein itself were present, as compared with when LexA repressor proteins were present (Fig. 5 and results not shown). This result is in agreement with previous results indicating that the scs element does not contain enhancer activity (7). We therefore conclude that the scs insulator is able to efficiently block repression of gene activity mediated by the chromatin-associated repressors HPC2, Su(z)2, RING1, mHP1, and MeCP2.

The Gypsy Insulator Selectively Blocks Chromatin-associated Repressors—We cloned the 0.4-kbp gypsy insulator (11) in both orientations between the LexA operators and the heat shock-
inducible promoter (top of Fig. 6). We found that in the presence of the gypsy insulator, the LexA-Su(z)2, LexA-RING1, and LexA-mHP1 proteins were unable to repress the heat shock-inducible promoter (Fig. 6). No difference was observed whether the gypsy insulator was cloned in the 5' to 3' orientation or the 3' to 5' orientation. Surprisingly, in the presence of the gypsy insulator, both the LexA-HPC2 and LexA-MeCP2 were still able to repress the reporter gene, independently of the orientation of the gypsy element (Fig. 6). Similar to scs, we cloned the gypsy insulator in the 5' to 3' orientation between the LexA operators and the HSF-inducible promoter, and (iv) the 1.7-kbp scs insulator in the 5' to 3' orientation cloned upstream of the LexA operators (bottom). Transfections, quantification, and representation of the data are as in Fig. 3.

Finally, the inability of gypsy to block repression that is mediated by the LexA-HPC2- and LexA-MeCP2-mediated repression could be due to an improper chromatin structure of the gypsy insulator. To exclude this possibility, we established LexA-HPC2-, LexA-Su(z)2-, LexA-RING1-, LexA-HP1-, and LexA-MeCP2-expressing cell lines in which the gypsy insulator-containing reporter construct was stably transfected. With any repressor protein, we observed a nucleosomal chromatin structure on the reporter gene, both 72 h after transfection (Fig. 2a, lane 1) and in the stably transfected cells (lane 2). We found that also in the stably transfected cell lines, gypsy was unable to block LexA-HPC2- and LexA-MeCP2-mediated repression (data not shown). In the stably transfected cells, we found that gypsy still was able to block LexA-Su(z)2-, LexA-RING1-, and LexA-HP1-mediated repression (data not shown). Therefore, we conclude that the gypsy insulator is able to block repression of gene activity mediated by the chromatin-associated repressors.
Chromatin-associated Repressors Are Blocked by Insulators

A Drosophila MAR/SAR Element Does Not Block Chromatin-associated Repressors—We placed a 1.0-kbp Drosophila histone MAR/SAR element (16, 29) in both orientations between the LexA operators and the heat shock-inducible promoter (top of Fig. 9). This particular 1.0-kbp portion of the MAR/SAR has the ability to bind to the nuclear matrix (29). We found that in the presence of the histone MAR/SAR element, the LexA-HPC2-, LexA-Su(z)2-, LexA-mHP1, and LexA-MeCP2 proteins were still able to efficiently repress gene activity (Fig. 9). No difference was observed whether the histone MAR/SAR element was cloned in the 5′ to 3′ orientation or in the 3′ to 5′ orientation. The inability of the MAR/SAR element to block the repression mediated by the LexA repressors could be due to an improper chromatin structure of the MAR/SAR element. To exclude this possibility, we also established LexA-HPC2-, LexA-Su(z)2-, LexA-mHP1-, and LexA-MeCP2-expressing U-2 OS cell lines in which also the MAR/SAR element-containing reporter construct was stably transfected. Similar to what we observed for the gypsy insulator, we found that the MAR/SAR-containing construct has a nucleosomal chromatin structure on the reporter gene, either 72 h after transfection (Fig. 2A, lane 2) or in the stably transfected cells (lane 4). Also in the stably transfected cell lines, we found that the MAR/SAR element was unable to block LexA-HPC2-, LexA-Su(z)2-, LexA-mHP1, and

Fig. 7. The 5′-HS has limited ability to block chromatin-associated repressors. The indicated LexA repressor proteins in the independent background U-2 OS cell lines were targeted to the LexA operators on three different reporter constructs. These reporter constructs consisted of (i) 1 kbp of λ DNA (top), (ii) the 1.3-kbp 5′-HS element in the 5′ to 3′ orientation, and (iii) the 5′-HS element in the 5′ to 3′ orientation (bottom) between the LexA operators and the heat shock-inducible promoter. Transfections, quantification, and representation of the data are as in Fig. 3.

Fig. 8. The core element of the 5′ DNase I-hypersensitive site blocks repression in an orientation-dependent fashion. The indicated LexA repressor proteins in the independent background U-2 OS cell lines were targeted to the LexA operators on three different reporter constructs. These reporter constructs consisted of (i) 2 kbp of λ DNA (top), (ii) the 1.7-kbp-long array of six 5′-HS core elements in the 5′ to 3′ orientation, and (iii) the long array of six 5′-HS core elements in the 3′ to 5′ orientation (bottom) between the LexA operators and the heat shock-inducible promoter. Transfections, quantification, and representation of the data are as in Fig. 3.
DISCUSSION

Drosophila Insulators Block Repression Mediated by Chromatin-associated Repressors—In this paper, we report that insulators or boundary elements are able to block repression of gene activity in human cells. We developed a repression system based on a human cell line in which we use an episomal reporter construct. This system has advantages over the commonly used Drosophila white reporter gene, in which the random integration of the reporter construct into the genome excludes easy control of that system. Our system is more easily controlled, both in terms of independence of position effects and the ability to select and test specific, well defined repressor proteins.

Our results show that several insulators are able to block repression mediated by the chromatin-associated repressors we used. Of these, the scs insulator was most efficient in blocking the repressors we tested (Fig. 4). This result demonstrates a striking evolutionary conservation, since the scs insulator is used outside its natural environment, Drosophila. This implies that there are human proteins that bind to the scs element in such a manner that the insulator becomes functional. Also, the function of the gypsy insulator is functionally conserved, since gypsy is able to block most of the repressors we used. However, unlike scs, gypsy was not able to block repression mediated by HPC2 or MeCP2 (Fig. 5). There are several explanations for this observation: (i) it is an intrinsic characteristic of the gypsy insulator; (ii) the DNA-protein interaction within the gypsy nucleoprotein complex is not sufficiently conserved to allow the insulator to function properly within the context of the human cell line; (iii) the gypsy insulator does not obtain a proper chromatin structure that allows the insulator to become fully functional. At present, our data do not favor the last two options. First, if the evolutionary conservation is insufficient, it is hard to explain why gypsy is very efficient in blocking repression mediated by RING1, Su(z)2, and mHP1. With insufficient conservation, one might expect that the gypsy insulator would block no vertebrate repressor at all. Secondly, to exclude an improper chromatin environment, we made stably transfected cell lines with the reporter construct that contains gypsy. Also, in that case gypsy was unable to block repression mediated by HPC2 and MeCP2. Furthermore, we detected no significant differences in the nucleosomal chromatin structure of the reporter construct containing gypsy. Finally, if an improper chromatin structure plays a role, this would not explain why, under similar conditions, is very efficient in blocking repression mediated by RING1, Su(z)2, and mHP1. When taking these arguments together, we favor the possibility that our results indicate that it is an intrinsic characteristic of the gypsy insulator to block repression by RING1, Su(z)2, and mHP1 but not by HPC2 and MeCP2. Apparently, gypsy is able to block chromatin-associated repressors with a high level of selectivity. This establishes an important point. Whereas both HPC2 and MeCP2 are able to very efficiently repress gene activity, they are different from the other repressors in the sense that their action cannot be blocked by the gypsy insulator. The assay we used thus uncovers both differences in the ability of insulators to block repression and differences between chromatin-associated repressors. The differences between repressors do not become apparent when only their abilities to repress gene activity are being monitored.

The Limited Abilities of the 5′-HS Element and a MAR/SAR Element to Block Repression—Our results show an orientation dependence of the 5′-HS element in the chicken β-globin locus. Whereas a single copy of the entire element did not have much effect on the repression mediated by any of the repressors tested, we found a distinct effect when a tandem of six core elements within the 5′-HS element was tested (Fig. 7). Previously, it has been shown that the enhancer blocking ability of the 5′-HS element resides precisely in this core element (13). Our finding that the tandem of 5′-HS core elements is very efficient in blocking repression, but only when cloned in the 5′ to 3′ orientation, came as a surprise. The fact that this was true for all repressors tested gives weight to the idea that this orientation dependence is an intrinsic property of the 5′-HS element. The possibility that this is a consequence of an evolutionary gap is excluded by the fact that, being derived from a vertebrate, the 5′-HS element is the most conserved element we tested.

Finally, we found no indication that a Drosophila MAR/SAR element is able to block chromatin-associated repressors. This was observed in a stably transfected cell line and in a cell line 72 h after transfection. In either case, we observed a bona fide nucleosomal chromatin structure. It should also be pointed out that the ability of MAR/SARs to shield reporter genes against enhancers and repressing chromatin is controversial (see below). When considering this together with our results, we conclude that the portion of the Drosophila histone MAR/SAR element we tested does not possess an ability to block repression.

The Ability of Insulators to Protect against Repression in...
Other Systems—The result that is most easy to interpret is the efficient blocking of repression by the scs insulator, since scs blocks any reporter we tested. How do these findings relate to previous studies? The scs and gypsy insulators have been tested previously for their ability to protect a reporter gene against position effects in transformed flies (6, 7, 32). The white maxigene construct is able to confer high expression levels of white and is prone to repression due to position effects. The white maxigene was flanked with the scs and scs’ elements (6, 7). The elements conferred a consistently high level of white expression in the majority of transformants, independent of the integration position within euchromatic regions of the genome (6, 7). These results strongly suggest that scs and scs’ are able to efficiently block repression. Our results, demonstrating the ability of the scs element to block repression, are in agreement with these earlier studies.

The gypsy insulator has not been tested in the context of the white maxigene. Instead, the gypsy insulator was used to flank the white minigene (32). These constructs are considered to be easily affected by position effect variegation, a phenomenon that involves repression in a heterochromatin environment. The extent of position effect variegation increased when these constructs were tested in fly lines that lack functional Su(Hw) protein. Su(Hw) is the protein that binds to gypsy and is necessary for gypsy to function properly (32). These results have been interpreted as indicating that in these fly lines the gypsy insulator does not function properly and that, consequently, repression was blocked less efficiently (32).

The 5′-HS element has been tested in Drosophila embryos within the context of the white minigene. In this assay, the ability of the 5′-HS element to protect against activation emerging from the surrounding chromatin was tested, not the ability to protect against repression. However, recently the 5′-HS element was found to convey position-independent expression levels to a reporter gene that was stably integrated in a chicken cell line (14). This favors a model in which the 5′-HS element protects a reporter gene against both activating and repressing influences emerging from the surrounding chromatin.

The effects of MAR/SAR elements that flank a reporter gene on its activity are controversial. Whereas position dependence of reporter gene expression has been claimed (15, 33), other reports claim that MAR/SAR elements induce a higher gene activity per se, but not position-independent expression levels (17). On the other hand, particularly in plants, MAR/SAR elements have been shown to induce position-independent expression levels (34). This indicates that insulation activities may co-localize with some MAR/SAR elements. However, in our particular repression system, we found that MAR/SAR elements were unable to block any chromatin-associated repressors we tested.

Previous Evidence for a Relationship between PcG Proteins and Insulators—What is the evidence from other studies that link PcG proteins to the function of insulators? The most convincing genetic evidence indicates that the function of PcG depends on PcG-mediated repression (10). Mutations in PcG genes suppress the insulator properties of gypsy, as monitored by its ability to prevent enhancer-promoter interactions (10). It has further been found that when either gypsy or scs is placed between a polycomb response element and a promoter, the repression initiated from the polycomb response element is blocked (35, 36). Our data are in agreement with these earlier studies. Whether this also implies that insulators such as scs and gypsy function as stop signals to terminate spreading of the PcG complex remains speculative. Taken together, however, all data point toward an important role of PcG proteins in the function of the gypsy and scs insulators.

No published data are available that indicate an involve-

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