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The effect of matrix attached regions (MAR) and specialized chromatin structure (SCS) on the expression of gene constructs in cultured cells and in transgenic mice

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Abstract

The flanking sequences of several genes have been shown to direct a position independent expression of transgenes. Attempts to completely identify the insulating sequences have failed so far. Some of these sequences contain a matrix attached region (MAR) located in the flanking part of the genes. This article will show that the MARs in cultured cells located in the 3′ OH region of the human apolipoprotein B100 (Apo B100) and within the SV40 genome were unable to stimulate and insulate transgene expression directed by the promoters from a rabbit whey acidic protein (WAP) gene or from human cytomegalovirus (hCMV) early genes. In transgenic mice, the MAR from the Apo B100 and SV40 genes did not enhance the expression of a transgene containing the rabbit whey acid protein (WAP) promotor, the late gene SV40 intron (VP1 intron), the bovine growth hormone (bGH) cDNA and the SV40 late gene terminator. This construct was even toxic for embryos. Similarly, the specialized chromatin structure (SCS) from the Drosophila 87A7 HSP70 gene reduced chloramphenicol acetyl transferase (CAT) activity when added between a cytomegalovirus (CMV) enhancer and a Herpes simplex thymidine kinase (TK) gene promoter. This inhibitory action was almost complete when a second SCS sequence was added before the CMV enhancer. Sequences from the firefly luciferase and from the human gene cathepsin D cDNA used as control unexpectedly showed a similar inhibitory effect when added to the CMVTKCAT construct instead of SCS. When added before the CMV enhancer and after the transcription terminator in the CMVTKCAT construct, the SCS sequence was unable to insulate the integrated gene as seen by the fact that the level of CAT in cell extracts were by no means correlated with the number of copies in individual clones. From these data, it is concluded that i) a MAR containing the canonical AT rich sequences does not amplify the expression of all gene constructs ii) AT rich MAR sequences do not have per se an insulating effect iii) Drosophila SCS from the 87A7 HSP70 gene has no insulating effect in all gene constructs (at least in mammalian cells) iv) and the addition of a DNA fragment between an enhancer and a promoter in a gene construct cannot be used as a reliable test to evaluate its insulating property.

Introduction

Numerous experiments have shown that the expression level of foreign genes in mice is largely unpredictable and highly dependent on their integration site. However, in some cases, the gene constructs are expressed in an integration site-independent manner, thereby rendering, the expression level of the transgenes dependent on their copy number. This was the case for the human histocompatibility complex class I genes [1], the human CD2 gene [2], the human adenosine deaminase [3], the mouse metallothionein gene [4], the human keratin K18 gene [5], the rat LAP (C/EPB

β) gene [6], the rat aldolase gene [7] and the mouse β-myosin heavy chain [8].

Attempts to identify the sequences responsible for this insulating effect have not led to clear conclusions so far. Similarly, experiments aiming to elucidate the mechanisms generating the insulation of endogenous genes or of transgenes have given only partial explanations. In the case of the chicken lysozyme gene, elements located far in the upstream and downstream regions of the gene have been shown to be necessary for the insulation of the transgene [9]. The upstream region appeared to be sufficient for insulation when added on both sides of a reporter gene in cultured cells [10]. A matrix attached region (MAR) has been found in the upstream region of the chicken lysozyme gene [9]. The binding of a chromatin region to the nuclear matrix is generally considered to be correlated to the expression of the genes located in this region, although the nuclear matrix is still not a clearly defined biochemical entity [11]. The insulating effect of the chicken lysozyme gene upstream region was originally attributed to the capacity of the MAR sequence to bind the transgenes to the nuclear matrix [9]. More recent studies have shown that several elements contribute to the generation of the insulating property of the lysozyme gene upstream region [12, 13]. Moreover, the insulating effect, or at least part of this effect, is not restricted to the lysozyme gene, as observed when chloramphenicol acetyl transferase [14] and the β-glucuronidase genes [15] were used in transgenic mice and tobacco, respectively.

An AT rich MAR located within an intron seems essential to allow a high expression of an immunoglobulin K gene in transgenic mice [16]. In the β-globin locus, MAR sequences are spread all along the 80 Kb [17]. The second intron of the β globin gene contains a MAR of unknown fonction [17]. In tobacco, an AT rich MAR sequence no longer than 325 bp is sufficient to insulate a transgene [18]. DNA sequences in the upstream and downstream regions of the human interferon β gene stimulate the expression of integrated genes in cultured cells [19] and in transgenic mice [20]. The upstream region contains a MAR sequence [21] which is believe to contribute to the stimulatory effect. A more direct demonstration has been given by gene constructs containing several copies of the consensus AT rich sequence found in most of the MAR studied so far [22, 24]. This AT rich sequence was shown to stimulate strongly the expression of an integrated gene construct containing the SV40 promoter and the luciferase reporter gene [25].

Finally, it should be mentioned that MARs are present in genomes from viruses such as the bovine papillowa virus [26] SV40 [27] and the Epstein Barr virus [28]. Interestingly, the MAR found in the Epstein Barr virus genome is not AT and its sequences do not ressemble that of other known MAR sequences. The β globin locus is bordered by locus control regions (LCR) which act as specific enhancers and insulators for the different globin genes of this locus. Several elements of this LCR take part in the insulation and enhancer effect [29–32]. The HS4 and HS5 region from chicken [33] and human [34] respectively have been shown to have strong insulating properties. This fact was established by experiments in which the insulating region was inserted in gene constructs between the LCR enhancer and a reporter gene.

In Drosophila, specialized chromatin structures (SCS) surrounding the 87A7 heat shock locus are capable of insulating transgenes [35]. These SCSs are AT rich but have not the capacity to bind to a nuclear matrix. They were also shown to induce a redistribution of topoisomerase II [36]. Similarly, the MAR sequence of the Drosophila histone locus induces a redistribution of histone H1 and topoisomerase II generating a chromatin opening [37, 38].

From these data, it seems impossible presently to define which DNA sequence constitutes an insulator and which mechanisms are involved in gene insulation. As far as MAR sequences are concerned, it seems only that MAR sequences are relatively numerous in genomes [39] but that, perhaps, some of them may participate in the insulating effect. It is also presently not clear if MARs alone can be insulators or if they have to be associated with other regulatory sequences.

The present work has been undertaken to tentatively evaluate the possible stimulatory and insulating effect of the MAR which is located in the 3′ OH region of the human apolipoprotein B100 (Apo B100) gene and which contains a large number of the consensus AT rich region [40], the SV40 MAR and the Drosophila SCS from the heat shock (HSP70) gene locus. For this purpose, the rabbit whey acidic protein regulatory region which is capable of directing a high expression level of foreign genes in the mammary gland of transgenic mouse [41] and the CMV enhancer region [42] respectively associated to bGH or CAT reporter genes were used. The gene constructs were tested in cultured cells using transient and stable assays and in transgenic mice.

Materials and methods

Gene construction

The matrix attachment region located in the 3′ OH region of the human apolipoprotein B100 (Apo B100) [40] was obtained by PCR and cloned in the SmaI site of pPoly III plasmid. The SmaI-HpaI fragment of the RSV luciferase plasmid [43] containing the SV40 MAR was excised and cloned in the PvuII of polylinker of pPoly III plasmid.

The rabbit WAP gene promoter 6.3 Kb [44] cloned in pPoly III plasmid was associated with the pSVL (Pharmacia) KpnI-XhoI fragment containing the SV40 late genes intron, a 5′ P UTR sequence, the bGH cDNA and the blunt BamHI-SalI fragment from pSVL containing the SV40 late gene terminators: (p14).

The MAR located in the 3′ OH side of the Apo B100 gene was introduced into the BamHI site of the plasmid p14 before the WAP promotor and 2 SV40 MARs in tandem were added into the SalI site after the SV40 late genes terminator (p34). In plasmid p8, one SV40 MAR was introduced in the SalI site of p14. The human cytomegalovirus (CMV) early gene promoter was introduced into the PvuII site of the pSVL vector (Pharmacia). A 5′ P UTR and the bGH cDNA were introduced into the XbaI and the SmaI sites of the polylinker located after the VP1 intron, respectively. The EcoRI-SalI fragment of this plasmid was exicsed and cloned in the polylinker of pPoly III generating the plasmid p13.

The MAR from the Apo B100 gene was introduced into the EcoRV site of VP1 intron of plasmid in p13 either in 5′ P–3′ OH (plasmid p22) or in 3′ OH–5′ P orientation (plasmid p23). The SV40 MAR was introduced into the same site in plasmit p13 in the 5′ P–3′ OH (plasmid p21) and 3′ OH–5′ P orientation (plasmid p20).

The BamHI-BamHI fragment containing the human cytomegalovirus early gene enhancer from the pCMVTKCAT plasmid [45] was cloned in the EcoRV site of the Bluescript plasmid. The XbaI-PvuII fragment was isolated from this plasmid, blunted and finally cloned in the pCMVTKCAT in the upstream of the TK promoter generating the plasmid p91 with two unique sites, a BamHI in the 3′ OH end and a BssHII on the 5′ OH side of the CMV enhancer respectively.

The specialized chromatin structure (SCS) from theDrosophila 87A7HSP70 gene was obtained by PCR from Drosophila DNA and cloned in the pGEMT vector (Promega). This SCS was cloned in the plasmid p91, in the BamHI site, either in the 3′ OH–5′ P (p94), or the 5′ P–3′ OH orientation (p95) and in the BssHII site in either the 3′ OH–5′ P (p96) or 5′ P–3′ OH orientation (p97).

The SCS was also added on both sides of the hCMV enhancer (p98).

In the plasmid p99, finally, one SCS was present in the BssH II site of the plasmid p91 in 3′ OH–5′ P orientation and another SCS was added in the SalI site after the terminator, in the 5′ P–3′ OH orientation.

Two controls plasmids were constructed. The plasmid p104 contained the 1.6 kb firefly luciferase gene the (XbaI-SmaI fragment RSV luciferase added in the BamHI site of the plasmid p91). The plasmid p105 contained the 1.7 kb human cathepsin D cDNA [46] which was cloned in the BssHII site of the plasmid p104.

Cell transfections

In all cases, transfections were carried out using lipofectamine (BRL) as suggested by the manufacturer.

In transient expression assays, cells were collected 2 days after transfection and bGH and CAT were measured in medium and cell extracts respectively.

To evaluate the efficiency of the gene constructs containing the rabbit WAP gene promoter, stable mouse mammary HC11 clones were obtained after a co-transfection with the RSV-neo^r selection plasmid. Two independent dishes were transfected in all cases. The whole clones in each dish were amplified. The cells were then cultured in the presence of EGF (10 ng/ml) until hyperconfluency was reached. Lactogenic hormones (insulin 5 μg/ml, dexamethasone 10^{-6} M and ovine prolactin 5 μg/ml) were then added for two days in the absence of EGF. bGH concentration was measured in the medium. All these methods have been described in previous work [47].

Stable HC11 clones harbouring the gene constructs p91, p94, p98 and p105 were obtained using lipofectamine and RSV neo^r as depicted above. CAT assays were carried out on whole or in individual clones without any prior hormone action.

Transfections of CHO, COS and EPC were carried out as depicted above and as depicted in previous studies [47, 48].

Chloramphenicol acetyl transferase (CAT) assays

Cellular extracts were assayed for CAT activity using a mixed phases method. The complete reaction mixture (25 μl final volume) contained 15 μl of cell extracts

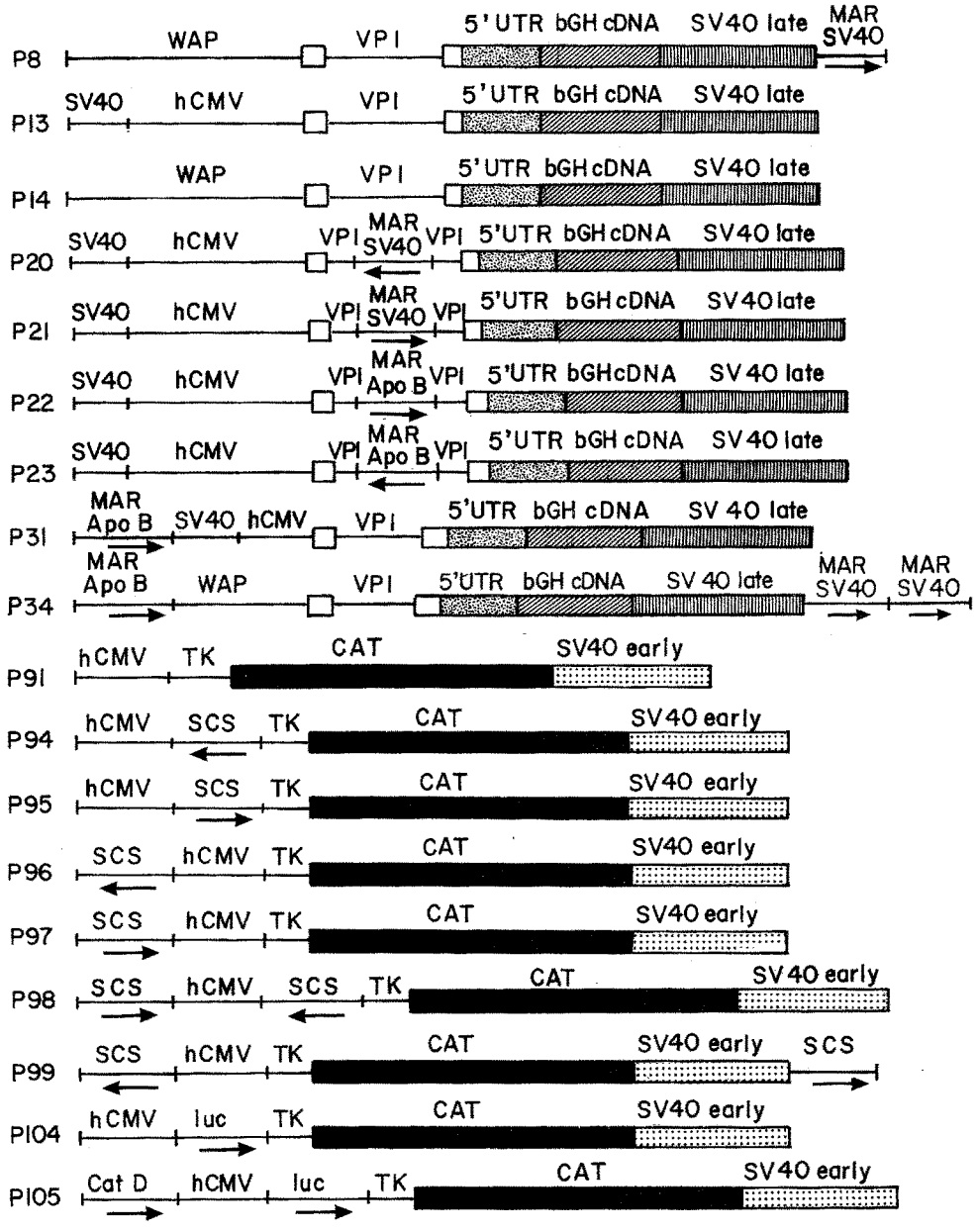

Figure 1. Schematic representation of gene constructs. The details of the gene construction are given in Materials and methods. The various gene fragments are as follows: hCMV: promoter and enhancer from human cytomegalovirus early genes; SV40: enhancer (without CAP site) from SV40 early genes; VP1: intron from SV40 late genes; 5′ UTR: an untranslated region which will be described elsewhere; bGH cDNA: bovine growth hormone cDNA; SV40 late: terminator from SV40 late genes; MAR SV40 a fragment of SV40 genome which contains a matrix attached region and the small t intron from early genes [27]; MAR ApoB: the matrix attached region located in the dowstream of the human apolipoprotein B100 gene [40]; WAP: regulatory region located in the upstream of the rabbit whey acidic protein gene [44]; TK: promoter region from the Herpes simplex thymidine kinase gene; CAT: chloramphenicol acetyl transferase gene; SV40 early: terminator from SV40 early genes; SCS: special chromatin structure from Drosophila 87A7 heat shock 70 locus [35]; Luc: firefly luciferase gene [43]; cat D: human cathepsin D cDNA [46]. The arrows indicate the orientation of the DNA fragment referred to in the direction of transcription. The different DNA fragments in the figure are not on scale. All the constructs were inserted into the pPoly III1 plasmid [56].

and 0.1 μCi of [^{3}H] acetyl-coenzyme A (5 Ci/mM, Amersham) in the presence of 30 μM acetylcoenzyme A, 1 μM chloramphenicol, 4 mM EDTA and 200 mM Tris pH 7.8. Incubation was performed for 2 h at 37 °C. The reaction was terminated by adding to the reaction mixture 2.5 ml of 7 M urea in a 5 ml scintillation vial. A Scintillation cocktail (2.5 ml) was added and radioactivity was determined using a scintillation counter as described [49].

bGH measurement

Concentrations of bGH in the culture media and in milk were determined in duplicate using a specific homologous bGH radioimmunoassay (NIH) as previously described [47].

Southern blot analysis

Genomic DNA (5 μg) prepared from scrapped cells by a genomic DNA kit (Kontron Instrument) was restricted by EcoRI and the fragments were separated according to their size by agarose gel electrophoresis, blotted onto nitrocellulose filters and hybridized with a DNA fragment labelled with ^{32}P-dCTP.

Results

The effect of MAR from SV40 and Apo B100 gene on gene expression level

The MAR located in the downstream region of the human Apo B100 gene [40] contains a particularly large number of the AT rich consensus sequence found in many DNA regions binding the nuclear matrix [22]. This MAR was added to the upstream of the rabbit WAP gene regulatory region. In several studies, it was reported that two MARs surrounding the gene constructs are needed to completely insulate the foreign integrated DNA. One or two copies of the MAR from SV40 genome was thus added after the terminator of several constructs (p8 and p34) (Figure 1).

The presence of these MARs in the borders of these gene constructs did not alter significantly the expression of bGH cDNA (Figure 2). The same observations was done with similar constructs containing human cathepsin D and human erythropoietin cDNAs instead of bGH cDNA (data not shown).

The effect of the MAR from the Apo B100 gene was also evaluated using constructs in which a hCMV promoter was used to direct the expression of bGH cDNA. The results illustrated in Figure 3 indicate that

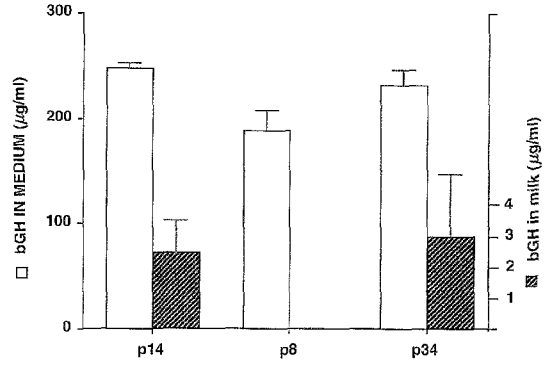

Figure 2. Effect of Apo B100 gene and SV40 MAR on the expression of bGH cDNA driven by the rabbit WAP gene promoter. The plasmids p8, p14 and p34 were transfected into the mouse mammary cell line HC11. Mixtures of stable clones at hyperconfluency were induced by lactogenic hormones (insulin, dexamethasone and prolactin) for two days. Results expressed as ng of bGH per ml of culture medium are the means of duplicates from three independently transfected dishes for each plasmid. The concentrations of bGH in milk are the means (± SEM) of duplicate from several independent transgenic mouse lines: 4 mice for the construct p14 and 5 lines for the construct p34.

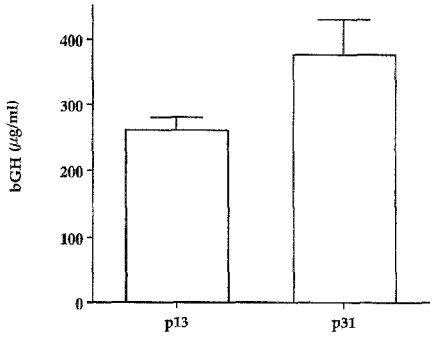

Figure 3. Effect of the MAR from the Apo B100 gene on the expression of bGH cDNA driven by the hCMV promoter. Plasmids p13 and p31 were stably transfected in HC11 cells using pRSV neo^r as a selection gene. Three independent dishes were transfected with each construct. The whole clones in each dish were amplified together and bGH was measured in the medium of reseeded cells. Results are the means of duplicate ± SEM of three independent dishes.

the MAR enhanced the action of the hCMV promoter in the stable HC11 clones modestly but significantly.

In several genes, a MAR is present within an intron. This is the case for mammalian β-globin gene [17]. The role of such a MAR, if any, is not known. The MARs from the Apo B100 gene and from SV40 genome were added in the VP1 intron of gene constructs containing the CMV promoter (plasmids p21, p22, p23 and p24). In no case did the MARs enhance bGH cDNA expression in stable CHO clones (Figure 3). Construct p20 was significantly less efficient than the others. This

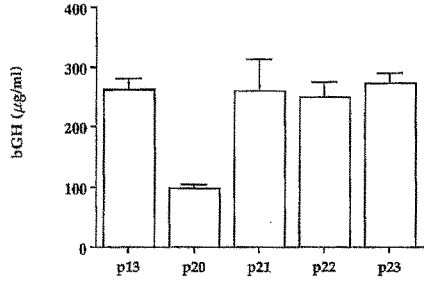

Figure 4. Effect of MAR from SV40 and from the human Apo B100 gene inserted into intron. The MARs were introduced in the EcoRV site of the VP1 intron in both orientations in constructs driven by the hCMV promoter (p20, p21, p22, p23). These constructs and the control p13 were transfected with pRSV neo^r into CHO cells. Three dishes were transfected independently with each construct. All of the entire clones resistant to geneticin were amplified together. bGH was measured in the culture medium of the clones reseeded simultaneously in new dishes. Results are the means of duplicate ± SEM obtained from three independent dishes for each construct.

may be due to the presence of the intron of SV40 early genes introduced with the SV40 MAR within the VP1 intron. Splicing signals of both introns may interfere and reduce the efficiency of the pre-mRNA maturation.

The gene constructs p14 and p34 were also used to generate transgenic mice. Data shown in Figure 2 indicate that the addition of MARs to both sides of the construct did not enhance the expression of the transgenes at all. On the other hand, for unknown reasons, all the gene constructs containing the MAR from the Apo B100 gene were highly toxic for embryos, and the concentration of injected DNA had to be reduced three times in order to obtain transgenic animals.

The effect of MARs from SV40 and the Apo B100 gene on the dependency towards gene copy number

In order to determine whether the MARs can provide a dependency towards the copy numbers of the integrated genes, the construct p34 was used to generate independent stable HC11 clones. The number of integrated copies and the level of bGH secretion were measured independently for 14 clones. Data in Figure 5 clearly indicate that no correlation was observed between the copy number of the integrated gene and the level of bGH secretion.

The effect of Drosophila SCS

Several loci in Drosophila are bordered by DNA sequences which show insulating properties when added to transgenes. Such sequences, named SCS and SCS' and located in the HSP 70 gene locus, have been studied intensively [35, 36].

A recent work reported that an insulating property of a DNA sequence can be identified by introducing this sequence between the enhancer and the promoter of a gene construct [33, 34].

The SCS from the Drosophila HSP70 locus was inserted between the CMV enhancer and the TK promoter of the pCMVTKCAT construct (plasmid 94). This SCS reduced the efficiency of the construct in stable clones (Figure 6) by half. The SCS sequence showed the same inhibitory activity when inserted in the opposite orientation (p95) (data not shown). The same result was obtained when the SCS was inserted before the hCMV enhancer (plasmids p96 and p97) (not shown). Two SCSs were also added in 5′ and 3′ sides of the hCMV enhancer to potentially isolate the gene from the CMV enhancer action (plasmid p98). The effect of the hCMV enhancer was then almost completely abolished (Figure 6).

Control DNA sequences not expected to have insulating properties were inserted in the CMVTKCAT construct. The firefly luciferase cDNA reduced the efficiency of the construct as strongly as the SCS (plasmid p104) (not shown). Unexpectedly, the cathepsin D cDNA inserted upstream of the hCMV enhancer in plasmid 104, in addition to luciferase cDNA (plasmid p105), abolished the expression of the CAT gene (Figure 6). The insulating effect of SCS cannot, therefore, be considered specific.

The insulation of a gene can be estimated by its capacity to be expressed in an integrated state as a function of the copy number. The gene construct containing two SCS sequences on both sides of the CMVTKCAT (p99) was transfected into CHO cells. Stable individual clones were examined in order to compare the level of CAT gene expression and the number of CAT gene copies. Results of Figure 7 show clearly that the level of CAT gene expression was by no means correlated with the number of CAT gene copies in the different clones. These data do not support the idea that the Drosophila HSP70 SCS sequence has an insulating activity in mammalian cells.

Conclusion

Gene insulation is probably an essential mechanism controlling gene expression. It is also essential for transgenes which, most of the time, are expressed in a non reproducible manner and independently of their copy number. This is particularly important for transgenes expressed in the mammary gland and for direct-

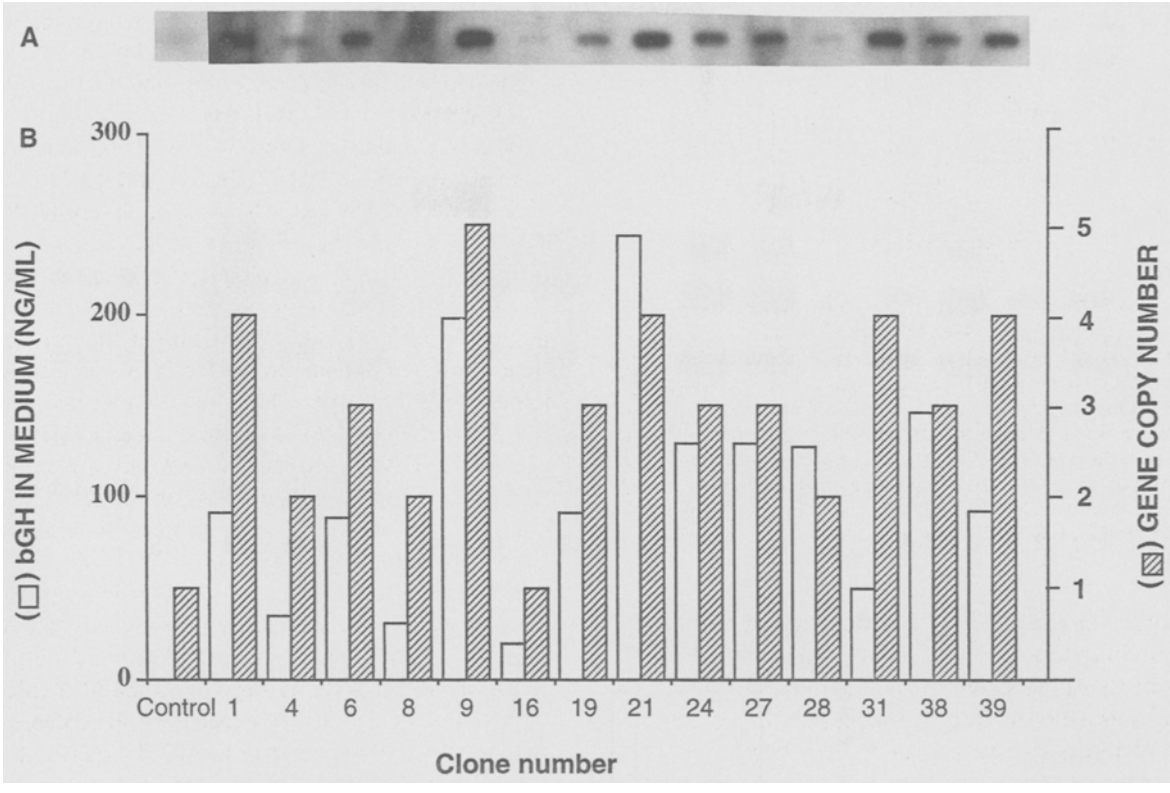

Figure 5. Effect of MARs on the dependency towards copy number of integrated genes. The plasmid p34 was used to obtain stable HC11 independent clones. The number of integrated copies was evaluated by Southern blotting. One of the bands generated by a digestion of total cellular DNA with ScaI is shown in A). A comparison of gene copy number and bGH concentration for 14 clones is hown in B). In all cases bGH was measured in duplicates after 2 days of stimulation by lactogenic hormones.

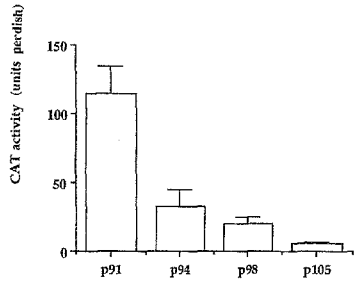

Figure 6. Effect of the SCS from Drosophila 87A7 heat shock locus on the activity of hCMV enhancer in HC11 cells. The SCS sequence was inserted on both sides of the enhancer (see Figure 1). The firefly luciferase gene and the human cathepsin D cDNA were used as control DNA sequences. A pool of stable HC11 clones was obtained in two independent dishes for each construct. Results are the means of duplicate from two independently transfected dishes. The CAT activity represents the total activity of the cell extracts in each dish. The numbers refer to the gene constructs depicted in Figure 1.

ing the expression of recombinant protein of pharmaceutical interest [50]. Indeed, to be successfull, this method must ideally lead to a high level of secretion of the foreign proteins in milk and to an expression of the transgenes exclusively in the mammary gland. In some cases, the ectopic expression of transgenes alters the health of animals. This was the case for the human erythropoïetin gene which dramatically increased the number of red blood cells in transgenic mice and rabbits even when controlled by the rabbit WAP gene promoter which works only in the mammary cells in its native state (unpublished data).

The experiments reported here indicate that the 3′ OH MAR from the human Apo B100 gene, although containing a large number of the AT rich consensus sequence, was completely unable to enhance the efficiency of the rabbit WAP gene regulatory region, and stimulated the hCMV regulatory region only modestly. The repeated AT rich consensus sequence found in MAR is a very strong transcription stimulator for the SV40 promoter [25, 51].

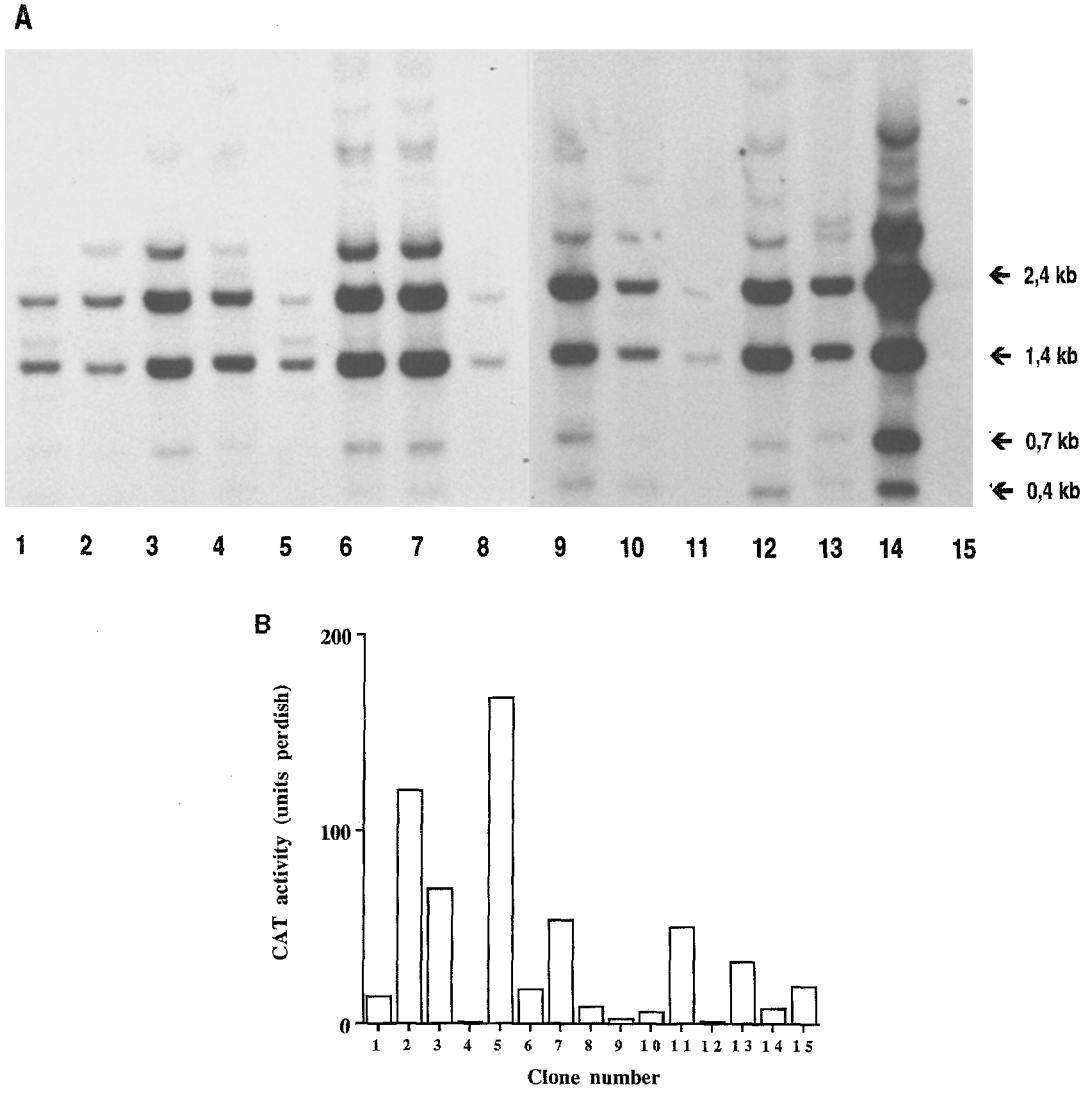

Figure 7. Measurements of the insulating effect of the SCS region from the Drosophila 87A7 heat shock locus. The SCS region was inserted in both sides of the gene construct p91. The resulting gene construct (p99) was transfected into HC11 in the presence of the pRSV neo^r plasmid. Clones resistant to geneticin were grown separately. The number of CAT gene copies and the level of CAT gene expression were measured in the different clones. Results of CAT activity are the means of duplicates from each clone extracts. The copy number of the CAT gene in each clone was obtained after Southern blotting analysis, using 10 μg of total cellular DNA restricted by EcoRI and the whole ^{32}P labelled p99 plasmid as probe. A) Southern blotting B) CAT activity in the different clones. The numbers indicate the different individual clones harbouring the integrated p99 plasmid.

The results of present work indicate that this AT rich sequence cannot be considered a general transcription stimulator acting with all promoters. The SV40 promoter region generally used contains a relatively short fragment of the SV40 genome. The MAR present in the SV40 genome may participate in the action of the promoter. The experimental addition of the AT rich sequence to SV40 may in some way restore the natural situation. The hCMV regulatory region is known to be a very strong promoter and it may need to additional stimulator. The same may be true for the rabbit WAP promoter which is very strong when used in the mammary cell [41].

Regions bordering human β-interferon and chicken lysozyme genes have been shown to stimualte the expression of genes located in their vicinity [9, 19, 20]. These regions contain AT rich sequences but also long

DNA fragments which may participate in the stimulatory effect.

The MAR from the Apo B100 gene showed no insulating effect. This conclusion is in agreement with a recent study which showed that the Apo B100 MAR has no insulating action in vivo [52]. The Apo B100 MAR used in the present study is located in the downstream region of the gene [40]. It is conceivable that its function is not to favour the expression of the gene. Interestingly, however, a recent study [53] has shown that the MARs located in the 5′ P and the 3′ OH of the human Apo B100 gene participate in the insulation of the gene in hepatoma cells and in transgenic mice. It is conceivable that these MARs contribute to the insulation of the only Apo B100 gene expressed in liver cells.

The Drosophila SCS sequence reduced the enhancer effect of the CMV upstream region considerably when added before the TK promoter and before the hCMV enhancer. From this data, it might be concluded that the SCS used has kept its insulating activity in mammalian cells. The fact that the luciferase and cathepsin D cDNA sequences used as control exhibited exactly the same inhibitory effect casts some doubt on the idea that an insulating effect of a DNA sequence can be identified only by its capacity to interrupt the action of an enhancer. Indeed, the SCS sequence used was unable to express the pCMVTKCAT in a copy number dependent manner when added to both sides of the construct. The Drosophila SCS from the HSP locus seems therefore to have no insulating activity in mammalian cells.

From the present work and from other studies, it appears that gene insulation is a complex and most likely not unique mechanism [54]. Obviously, MARs alone are quite insufficient to induce an insulation. The fact that MARs have been found in several insulating regions such as in the lysozyme gene and the β-globin locus suggests that their participation is important in some cases. Interestingly enough, a recent study has shown that the MAR found in the upstream part of the human β-globin locus is able to insulate a gene in cultured cells when associated with the polyoma enhancer [55]. Hence, some of the insulating region might contain a non-specific AT rich MAR as well as transcription enhancers. The presence of these enhancers might help to give the gene construct a cell specificity to some of the insulating regions. Additional studies are necessary to determine if this hypothesis is valid.

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