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Scaffold-Attached Regions from the Human Interferon β Domain Can Be Used To Enhance the Stable Expression of Genes under the Control of Various Promoters†

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ABSTRACT: We have transfected DNA corresponding to the complete chromatin domain of human interferon β (huIFN-β) gene into mouse L cells. In this construct, which is flanked by scaffold-attached regions (SARs), the gene's transcription was enhanced 20–30-fold with respect to DNAs containing only the immediate regulatory elements. To elucidate the role of SAR elements in the transcriptional enhancement, their position was varied relative to several artificial promoter–gene combinations. It was found that SARs enhance general promoter functions in an orientation- and partially distance-independent manner; their effect is restricted to the integrated state of transfected templates. During the phase of transient expression, SAR elements were generally found to have an antagonizing effect.

The induction mechanism of the type I interferon (IFN) genes is understood in considerable detail [see Maniatis (1986) and Bode and Hauser (1990) for reviews]. All elements involved in this process have been localized within 200 and most of them within 100 base pairs of DNA upstream from the transcriptional start. This information about the immediate control region arises from "reverse genetics", which involves

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transferring the human gene together with selected regulatory sequences into mouse cells and monitoring the effects on interferon titers. For simplicity, these experiments were mostly performed during the phase of "transient expression", i.e., at a time prior to the template's integration into the host genome before the final chromatin structure has been established. Alternatively, selection procedures have been developed to screen for those transfectants which have received, integrated, and processed intact copies of a given construct. Only for the latter case can expression levels be referenced to the number of gene copies.

By adoption of such a "stable expression" approach, it was calculated that the level of IFN gene expression arising from the immediate regulatory sequences is low, usually in the order of 0.5–1% of the gene in its native environment. This situation could be significantly improved by the transfection of larger regions of human DNA, in which case a 10–20-fold increase of transcription was reported (Hauser et al., 1982; Collins, 1984).

Recent analyses of remote sequences revealed 7 kb of DNA 5′ to the human interferon β (huIFN-β) gene which contributed to scaffold association over their entire length; a corresponding 3′-element covered at least 3 kb (Bode & Maass, 1988). The murine counterpart (muIFN-β) was found to be organized in an analogous manner (Mielke et al., 1990). These attachment sequences, which are 14 and 20 kb apart, define the chromatin domains involving the IFN-β genes of humans and mice, respectively. Elements of this type have been classified as "scaffold-attached regions" ["SARs";[1] cf. Gasser and Laemmli (1987) and Gasser et al. (1989)] or "matrix-attached regions" ["MARs"; cf. Cockerill and Garrard (1986) and Blasquez et al. (1989)] by slightly different criteria. Applying the procedures used for their identification, the elements adjacent to the interferon genes would qualify both as SARs and MARs (Bode & Maass, 1988; Mielke et al., 1990) although the published MARs are mostly intronic elements which may serve a related but distinct function [cf. Mielke et al. (1990)].

The discovery of an interferon domain that is bordered by SAR sequences raised a number of relevant questions regarding the nature, the minimum extension, and the optimum positioning of these elements. In our initial work we analyzed the relative affinity of various SAR and MAR sequences to the nuclear scaffold in vitro and demonstrated a corresponding capacity for these elements to enhance the transcription in vivo (Mielke et al., 1990). In this set of subsequent experiments we used the most prominent elements of this type to investigate a number of more involved questions regarding the optimal distance of SAR elements from the transcriptional start, their position relative to the gene, and their promoter specificity. Moreover, their effects prior to and after integration were analyzed and correlated to SAR function.

Materials and Methods

(*a*) *Plasmids.* pAG60 (Colbère-Garapin et al., 1981) was modified to pAG60′ by eliminating the *Eco*RI site within the TK promoter by a partial digest, Klenow reaction, and religation; this alteration does not change the vector's ability to mediate resistance to the aminoglycoside G-418. For plasmid pAG·F the remaining *Eco*RI site (position 0) was used to clone the 1.9 kb *Eco*RI F fragment.

pAG·B′ was constructed by inserting the 14.2 kb *Bam*HI fragment (B′ in Figure 2) from pCosIFN-β (Gross et al., 1981) into the single *Bam*HI site of pAG60′. A partial *Bam*HI digest followed by Klenow treatment and religation resulted in a derivative into which the 1.8 kb *Bam*HI fragment (C′) of pCosIFN-β could be inserted downstream from the IFN gene to reconstitute 16 kb of the native arrangement (construct pAG·B′C′). Alternatively, the 4.3 kb *Eco*RI–*Hin*dIII fragment b was cloned between the corresponding sites of pAG60′ followed by insertion of the 14.2 kb *Bam*HI B′ fragment into the respective site to yield pAG·B′b. Similarly, the 11.7 kb *Eco*RI fragment A′ was cloned into the *Eco*RI site of pAG60′ followed by insertion of B′ to yield pAG·A′B′; A′ originated from a λ clone [C15; cf. Mory et al. (1981)] and provided the downstream SAR of the huIFN-β gene.

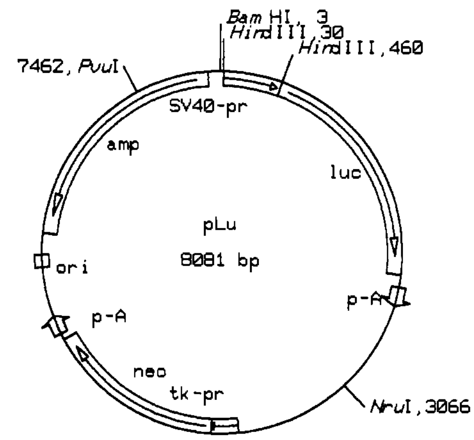

FIGURE 1: pLu, a plasmid for the study of SAR effects upon different promoters, using luciferase as a marker gene. In pLu, luciferae is under the transcriptional control of the SV40 early promoter; other promoters could be introduced via the *Hin*dIII sites. The *Bam*HI and *Nru*I single sites were used as acceptors for SAR elements. For transfection, the respective plasmid was linearized by using its *Pvu*I site.

The construction of the 10.1 kb plasmid pCATIFβ from sequences coding for human IFN-β, for bacterial CAT, and for resistance to the neomycin analogue G-418 (MoMSV-LTR/Nm^R) has been described (Klehr & Bode, 1988), and several of its applications have been reported (Wirth et al., 1988; Mielke et al., 1990). In the present context its nomenclature was changed to "pCI" to allow brief designations. The plasmid contains three *Eco*RI sites at positions 6097 (site a), 10100/0 (site b), and 3297 (site c). By use of two rounds of partial *Eco*RI digest/fill-in/ligation reactions, a set of plasmids was prepared, each member of which retained a single *Eco*RI site (pCIa, pCIb, and pCIc).

Several plasmids based on the luciferase gene as marker gene under the transcriptional control of the SV40 early promoter were derived from plasmid pPALu (Artelt, 1989): pLu (Figure 1) resulted from ligating the 4.7 kb *Pvu*I–*Sal*I fragment of pAG60′ (containing the Nm^R gene under the transcriptional control of the TK promoter) to the 3.4 kb *Pvu*I–*Tth*III1 fragment from pPALu; pLu·E was obtained by ligating the same 4.7 pAG60′ fragment to an analogous 5.8 kb fragment from a pPALu derivative which contained, in its *Bam*HI site, the 2.2 kb SAR fragment E; this *Eco*RI fragment had been adapted by linkers. Plasmids pLu and pLu·E lent themselves to further modification; i.e., promoters could be exchanged via the *Hin*dIII sites, and additional SAR elements could be cloned into the single *Nru*I site.

Promoter Exchanges. For plasmids pMMTV-LU and pMMTV-LU·E the 431 bp *Hin*dIII fragment of pLu (Figure 1) was replaced by the 1.4 kb *Hin*dIII fragment from pMTVdhfr (Lee et al., 1981). This fragment covers the

[1] Abbreviations: MAR, matrix-attached region; SAR, scaffold-attached region; Nm^R (neo^r), neomycin resistance gene (Tn5 aminoglycoside 3′-phosphotransferase).

segment between positions 139 and 1548 of the viral genome. pHIV-Lu and pHIV-LU·E were obtained similarly by inserting the 0.9 kb *NruI–HindIII* fragment of pLTR-Cat into the *HindIII* sites of pLu. pLTR-Cat contains the *NarI–HincII* fragment of ARV (Jones et al., 1986).

Additional SAR Element. A 1.8 kb *EcoRI* fragment upstream from the ST-LS1 gene (potato) was found, by the in vitro assay (Mielke et al., 1990), to behave as a strongly binding SAR. After fill-in, this element was cloned blunt-end into the *NruI* sites of either pLu or pPALu.

(*b*) *Cell Culture and Gene Transfer.* Mouse L cells and BHK-21 cells were grown as described (Bode et al., 1986). Transfections were performed as follows: On day 1, 3×10^5 cells in 5 mL of DME/10% FCS were seeded into a 25-cm^2 flask. On day 2, the medium was changed, and 4 h later 500 μL (1 μg) of the precipitated plasmid–DNA was added. For preparation of precipitates, 1 μg of *linearized, carrier-free* plasmid DNA, dissolved in 250 μL of 125 mM $CaCl_2$, was added dropwise, with mixing, to 250 μL of 50 mM HEPES (pH 7.1), 0.28 M NaCl, and 1.5 mM sodium phosphate. The precipitate was left for 1 h at room temperature before use.

In cases where the construct was devoid of a covalently linked selector gene, pAG60′ was cotransfected at 10% the amount used for the marker plasmid. The cells were left overnight before the medium was changed for 5 mL of DME/10% FCS (day 3). On day 4, the cells were seeded into a 75-cm^2 flask and provided with 15 mL of selective medium (DME/10% FCS) containing 700 μg/mL neomycin analogue G-418 for L cells and 1000 μg/mL for BHK cells. The medium was replaced at days 8 and 10. The clones were counted on day 12.

For transient expression experiments, 1.2×10^6 cells were transfected on a six-well plate using a calcium(2+) phosphate coprecipitate from 1 μg of the respective luciferase plasmid and 1 μg of a reference plasmid containing the human β-galactosidase gene under the control of the human β-actin promoter. The cells were washed 48 h after transfection and lysed in 500 μL of 0.25 M Tris-HCl, pH 7.5. Fifty-microliter aliquots of the extract were used to determine the β-galactosidase and luciferase activities, respectively. Deviations of β-galactosidase from an average value were taken to reflect the variability of the cells' competence and ability to take up and process DNA. They were evaluated by correlating luciferase light units to β-galactosidase activity.

(*c*) *Copy Numbers.* The average number of integrated copies obtained by the above transfection protocol was determined by quantitative Southern blotting on DNA from pools of 100–200 clones. For quantification, the signal obtained from the same number of human FS-4 cells served as a standard (experiments in Figures 2 and 3); a signal generated from the endogenous muIFN-β was used throughout as an internal reference. Representative results are given in Figure 2, showing that below a certain construct length values of 1.2 copies per cell were attained. Analyses of bordering fragments that were performed on individual clones demonstrated the presence of single-copy integration events in the majority (80%) of cases. For the remainder, restriction analyses and polymerase chain reactions (using primers that extended in divergent directions and hence lead to amplification only in the case of head-to-tail integration events) gave no indication of tandem integration, which is rather typical of conventional protocols in which circular plasmids are transfected in the presence of carrier DNA.

As an additional control, constructs pCI and pCI$_\alpha$·E (Figure 3) or pLu and pLu·E (Figure 4) were also transferred by electroporation (Mielke et al., 1990). The selection of recipient cells required that the marker (huIFN-β or luciferase) gene and the selector (neo^R) gene were covalently linked, suggesting the integration of single DNA molecules. This property was again confirmed by quantitative Southern blot analyses. The expression levels monitored with this gene-transfer technique were in agreement with those obtained by the above transfection techniques.

(*d*) *Marker Gene Assays.* (*1*) *Interferon.* The methods used for determining the inducible expression of huIFN-β were as described by Dinter and Hauser (1987). Induction of confluent monolayers of transformed L-cell pools was performed with DEAE-dextran plus poly(rI)·poly(rC) and the titers were determined on FS-4 cells after challenge with vesicular stomatitis virus. Titers were standardized to 10^6 cells and 1 mL of supernatant.

(*2*) *Luciferase.* Extracts were prepared from a defined number of cells and tested for luciferase activity according to Williams et al. (1989). Usually 10^5–10^6 cells were lysed in 300–1000 μL of extraction buffer. The light emission of 10–50 μL was determined as the 0–10-s integral and referenced to the cell number.

(*3*) *β-Galactosidase.* On 96-well plates, $^1/_{10}$ of the above cell extracts was mixed with 40 μL of 2 mg/mL *o*-nitrophenyl β-D-galactopyranoside in 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 50 μM KCl, 5 μM $MgCl_2$, and 0.5 mM 2-mercaptoethanol. The mixtures were incubated at 37 °C until a yellow color became visible. The reaction was stopped by adding 100 μL of 1 M Na_2CO_3 and the absorption (A_{405}) determined in a Flow Multiscan reader.

Results

The 14-kb Chromatin Domain around the huIFN-β Gene Constitutes an Optimal Environment for Its Expression. Most studies on the induced activity of huIFN-β have made use of the sequence information provided by the 1.8 kb *EcoRI* fragment F (see Figure 2). Earlier observations from our laboratory have shown that expression levels can be increased significantly, if the gene is transfected in the context of longer sequences of human DNA (Collins, 1984; Bode et al., 1986). The constructs in Figure 2 were based on the information previously obtained on the huIFN-β chromatin domain (Bode & Maass, 1988). Parts of this domain were available in two different vectors, i.e., pCosIFN-β (Gross et al., 1981) and C15 (Mory et al., 1981), which were combined in construct pAG·A′B′ to yield a 26 kb DNA segment closely resembling the native domain for huIFN-β. Transfectants harboring this construct expressed 1200 IFN units per 10^6 cells following a standard induction protocol with poly(rI)·poly(rC). Deletions within the domain led to a continuous decay of gene activity from 1200 over 325, 225, and 150 IFN units to 10 units for the SAR-free construct pAG·F (Figure 2). Together with the gene copy number data, these results allowed a preliminary estimate about the effect that could be expected from the appropriate alignment of IFN-B SAR elements.

All transfections were performed with linearized vectors in the absence of carrier DNA according to a protocol which has been optimized to yield low copy numbers (Mielke et al., 1990). Quantitative Southern blots were then used to show that the shorter constructs up to and including pAG·B′C′ yielded an average of 1.2 copies per cell. By the same criterion, an increase of the average copy number 1.4 and 3.4 was monitored for the longer constructs pAG·B′b and pAG·A′B′, respectively. Southern blots and an assay based on PCR (polymerase chain reaction) techniques gave no indication of the presence of head-to-tail inserts which are rather typical

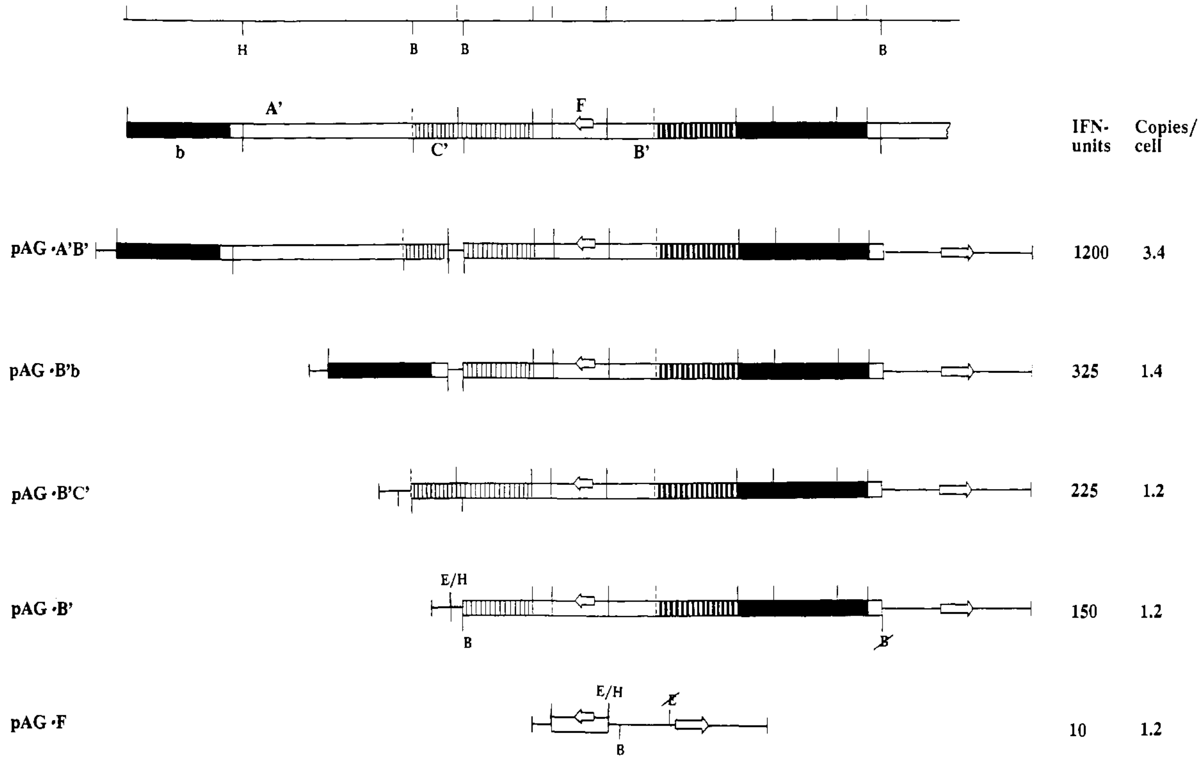

FIGURE 2: Reconstruction of the huIFN-β chromatin domain. The top two lines show a partial restriction map of the huIFN-β domain and a designation of the fragments used for the constructs. Bars above the lines, *Eco*RI sites; bars below the lines, *Hin*dIII (H) or *Bam*HI sites (B). The huIFN-β gene (arrow) is part of *Eco*RI fragment F. Beginning with line 2, affinities to the nuclear scaffold are classified by black or hatched boxes, indicating a 90–100%, 70–90% (solid pattern), and 40–70% (light pattern) association under standard conditions. Starting with line 3, plasmids are shown linearized at their single *Pvu*I site; this presentation approaches their state after integration into the chromatin of mouse L cells. The constructs are based on a pAG60 derivative providing the Nm^R gene under the control of the TK promoter; in this derivative (pAG60') the second *Eco*RI site (next to Nm^R) has been deleted. Constructs pAG·F, pAG·B', pAG·B'C', and pAG·A'B' represent continuous extensions of the domain while pAG·B'b is a "minidomain" with an internal deletion. Numbers to the right refer to expression levels (IFN units per 10^6 cells) and to the average number of integrated copies of huIFN-β (per cell).

for multiple-copy integration events (cf. Materials and Methods).

Since the highest expression levels had to be ascribed to a superimposition of direct SAR effects and copy numbers, a 20–30-fold transcriptional increase per single copy due to presence of SAR DNA could be anticipated. The following experiments were designed to explain these enhancement factors using a variety of similarly sized plasmids which yielded the same low copy number (1.2 per cell on average). Moreover, selected experiments were repeated using an electroporation technique which involved the transfer of linearized plasmids from a solution into the target cells. According to the protocol applied here, the technique required that the marker and selector genes reside on the same construct. It produced fewer clones (20–40 per transfer experiment) in which the single-copy level was strictly maintained.

Effects of the Nature and Localization of SAR Elements. We have constructed a set of plasmids, PCI_a, pCI_b, and pCI_c (Figure 3), which permit the insertion of SAR elements into either of three *Eco*RI sites at various distances relative to a marker gene (short arrow, huIFN-β) and to a selector gene (long arrow, Nm^R). An additional construct (pCI·B') was made by exchanging a 1.2 kb *Eca*I vector fragment for a 5.3 kb fragment of human DNA.

In the first group of experiments, human sequences upstream and downstream from the huIFN-β gene were extended in order to partially restore its natural environments. Construct pCI·B' (Figure 3, line 2) has a 4 kb stretch of native DNA downstream from huIFN-β due to the insertion of the *Eca*I fragment B'. The *Eco*RI subfragments of B' (D and G) have an intermediate (60–70%) propensity for an in vitro scaffold association (Bode & Maass, 1988), and this appears to be the reason why only a moderate enhancement of huIFN titers (from 10 to 30 units compared to the parent construct, pCI) was observed. The expression of the selector (Nm^R) gene is influenced in a similar way, as shown by mRNA measurements (Mielke et al., 1990) and by the number of clones arising from a standard transfection protocol (400 for pCI·B' as compared to 200 for pCI, see Figure 3).

For pCI_a·C, the appropriate *Eco*RI fragment of human DNA was inserted upstream from the transcriptional start into vector site "a". The 4.2 kb insert is fully scaffold-associated both by "in vivo" and "in vitro" criteria, and this is due to a considerable scaffold-binding potential in its distal *Pst*I subfragment (Bode & Maass, 1988). It is seen (Figure 3) that "C" raises IFN titers to a much higher extent than B', as expected from the relative SAR potentials. This is still true if fragment C is present in its inverse orientation, suggesting that the major attachment site can be moved to some extent without affecting the enhancement.

The same vector site (a) was chosen for construct pCI_a·E in which the most prominent SAR fragment of huIFN-β (E) was shifted closer to the gene. Again, the expression levels (120/100 IFN units, depending on orientation) correlated well with the fragment's scaffold binding potential in vitro (Mielke et al., 1990). To confirm that the 10-fold transcriptional increase for IFN-β was a function of scaffold-binding capacities, the effects of other strong SAR fragments in this position [as opposed to a non-SAR fragment of similar length and A+T content; cf. Mielke et al. (1990)] were compared.

Similar results were also obtained with SAR fragments C and E cloned into *Eco*RI site "b" downstream from huIFN-β,

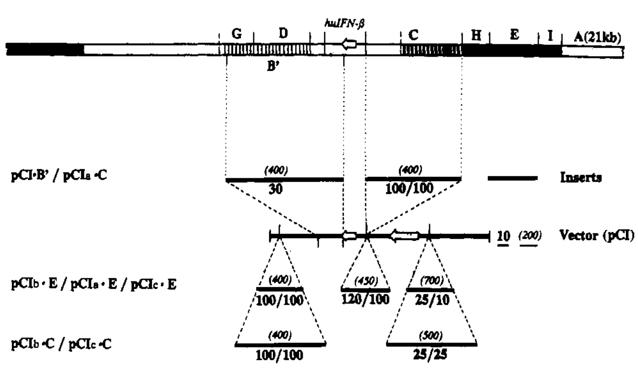

FIGURE 3: SAR effects depend on SAR strength and SAR position but are independent of SAR orientation. On top, the native huIFN-β domain has been depicted, and the various restriction fragments used for the present group of constructs have been indicated. The center line ["Vector (pCI)"] depicts the acceptor plasmid for these fragments, linearized via its *Pvu*I site. It includes a 0.96 kb *Eco*RI–*Bgl*II fragment around huIFN-β (short arrow to the left) and the Nm^R gene under the 5'-transcriptional control of the Moloney murine sarcoma virus LTR (right arrow). All subsequent data have been referenced to the average interferon titers (10 units) and clone numbers (200 per transfection) yielded by this construct. The line "inserts" shows the corresponding data (boldface number, IFN titer; italic number in parentheses, clone number) in a construct "pCI·B'" which reconstitutes 5.3 kb of DNA downstream from huIFN-β and in a separate construct (pCI·C) reconstituting 4.5 kb of human DNA upstream from huIFN-β. Our set of vector pCI derivatives offers *Eco*RI sites at positions "b", "a", or "c", respectively, which are located downstream from IFN-β, between the genes or upstream from Nm^R. These sites have been used for the insertion of fragments E or C at positions which differ from their native location. The corresponding constructs have been designated pCI_b·E, pCI_a·E, pCI_c·E, pCI_b·C, and pCI_c·C. All data refer to single-SAR constructs, i.e., constructs having the indicated segment of DNA inserted at the respective location. Fat numbers separated by a slash refer to either of two possible SAR orientations.

demonstrating a certain position independence of these elements (constructs pCI_b·E and pCI_b·C). The unexpected observation that the same SAR elements, cloned into the site "c" close to the 5'-end of the Nm^R gene, were rather inefficient in supporting IFN expression was later ascribed to silencing functions in the Nm^R coding region (see below). Since clone numbers (and mRNA levels) respond quite efficiently to a SAR element in site c, it can be concluded that the Nm^R gene itself profits from a nearby SAR sequence.

SAR Elements Become Effective Only after the Integration of Transfected Genes. After their transfer into the nuclei of eukaryotic cells, a burst of transcriptional activity can be monitored which is directed by templates prior to their integration into the genome (i.e., during the phase of "transient expression"). We compared the effect of SAR elements prior to and after the integration into the genome of the host cell. For convenience, the experiments used firefly luciferase as the marker gene, which enables a very sensitive assay and permits the detection of expression levels well below the range that can be assessed by IFN titers (Williams et al., 1989). Figure 4 summarizes a number of assays for plasmid pLu (Figure 1) and its derivatives, which include a covalently linked Nm^R gene. A parallel series of experiments has been performed in which the marker and selector functions were transfected on separate plasmids (pPALu and pAG60, respectively) in order to uncouple the coded functions and to avoid any potential cis-acting effects. Since both series led to essentially the same conclusions, only the data from the first series will be presented.

It is apparent from Figure 4 that SAR elements yield transcriptional enhancements whether they are positioned upstream or downstream from the marker gene, although to different extents. The 1.8 kb fragment "P" was originally derived from a plant gene (ST-LS1), the expression of which is position-independent after its transfer into potato and tobacco plants (Stockhaus et al., 1987), and it was found, by in vitro criteria, to be comparable to the 2.2 kb fragment "E" in its scaffold-binding affinity. The same is true for the transcriptional enhancements mediated by these elements if they are cloned into the same site (data not shown). Therefore, the higher value of pLu-P as compared to pLu-E is specific for this particular location which in all constructs was superior to the situation where the SAR element was positioned upstream from the luciferase gene. If both, "E" and "P" are used as flanking elements in a double-SAR construct, they cooperate to produce an almost 30-fold enhancement (cf. construct pLu-E-P).

SAR Sequences Enhance the Transciption from Various Promoters. The data presented in Figures 2 and 3 show that SARs improve the transcription directed by the virus-inducible huIFN-β promoter, and they strongly suggest that the same is true for the Moloney murine sarcoma virus (MoMSV) LTR which promotes the transcription from the aminoglycoside 3'-phosphotransferase (Nm^R) gene in vector pCI. The effects on the MoMSV are deduced from both the number of colonies obtained in a transfection experiment (Figure 3) and the corresponding mRNA levels (Mielke et al., 1990). Data in Figure 4 refer to the SV40 early promoter and are seen to conform with the other examples. Finally, we have exchanged the SV40 promoter in plasmids pLU and pLu·E for other promoters making use of the *Hin*dIII sites (Figure 1). Results for the mouse mammary tumor virus (MMTV) promoter in its (dexamethasone-) induced and quiescent states and for HIV promoter with and without activation by a coexpressed transacting (TAT) protein are presented in Figure 5. It is evident that the transcription is enhanced in all cases even by the presence of only a single SAR element, although to different degrees (3.5–10-fold).

Discussion

Scaffold-attached DNA regions (SARs) are operationally defined as elements with an intrinsic binding affinity for the nuclear scaffold or matrix. They are usually 300 or more base pairs long and about 70% A+T rich (Gasser & Laemmli, 1987; Gasser et al., 1989). Their binding is mediated by certain structural features which are only now beginning to be understood.

Many attempts to define a unique consensus sequence have essentially failed although a statistical overrepresentation of "Topo II boxes" (GTNA/TAT/CATTNATNNG/A as defined for *Drosophila*), an "A box" motif (AATAAAT/CAAA), a "T box" (TTA/TTT/ATTT/ATT), and stretches of ATATTT (together with its variants, ATATTTTT and AATATT) have been documented (Gasser & Laemmli, 1987; Cockerill & Garrard, 1986; Amati & Gasser, 1988; Mielke et al., 1990; Pommier et al., 1990). Structural features which have been implied in the recognition process include DNA single strands (Probst & Herzog, 1985), superhelicity (Tsutsui et al., 1988), bending (Homberger, 1989; von Kries et al., 1990), and a narrow minor groove due to oligo(dA) tracts (Adachi et al., 1989). We have recently demonstrated that all SAR sequences available to us have one property in common, i.e., the propensity of separating strands under the superhelical tension of a plasmid (Mielke et al., 1990; Kohwi-Shigematsu & Kohwi, 1990; Kohwi-Shigematsu and Bode, unpublished results). The nucleation center of this phenomenom coincides with an ATATTT-type motif, usually aatATATTT, which has been termed the "dominant tract". From this location, strand separation spreads to involve other tracts of this type which are evenly distributed over the entire length

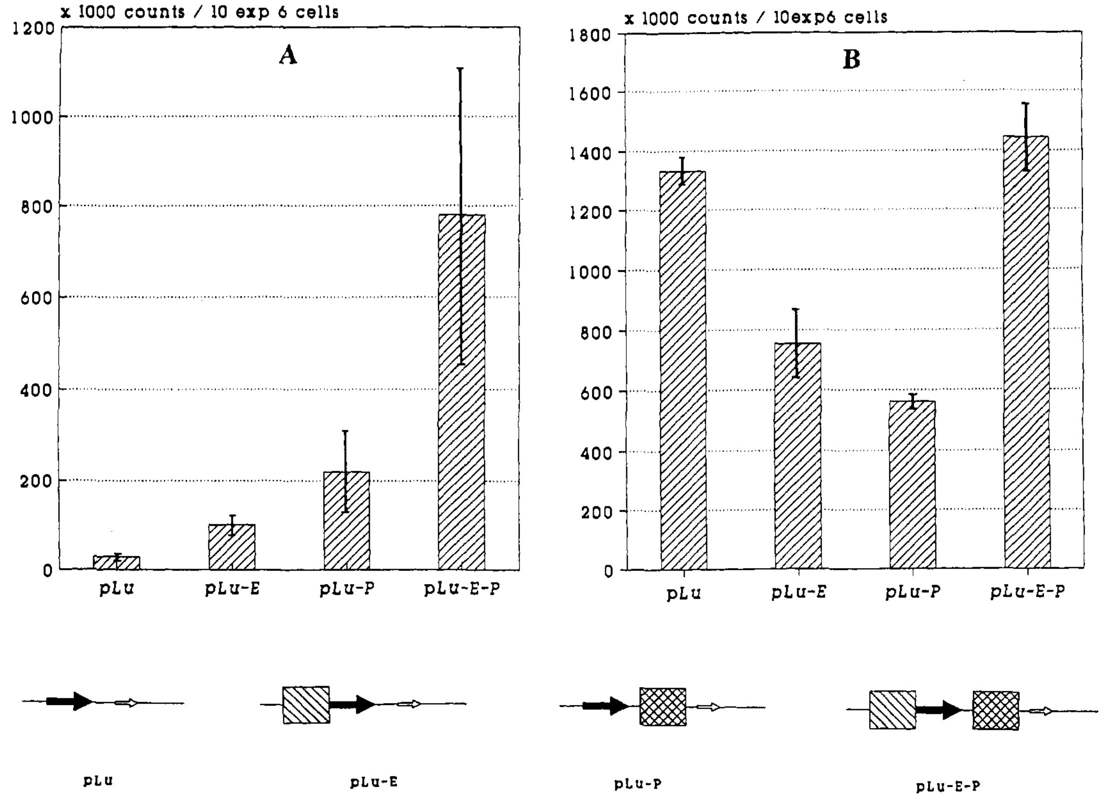

FIGURE 4: SAR elements enhance transcription only in the integrated state (conditions of stable expression). The constructs used for gene transfer by transfection and electroporation are depicted in the bottom line. For "pLu·E", pLu was provided with the 2.2 kb huIFN-β SAR fragment E (Figure 3) via its *Bam*HI site; "pLu·P", pLu with a 1.8 kb SAR fragment derived from a region upstream of the ST-LS1 plant gene, cloned into its *Nru*I site; "pLu·E-P", construct with both these SAR elements. (A) Luciferase activities (given as counts times 10^{-3} per 10^6 cells and 10 s) of plasmid pLu (Figure 1) and its derivatives. Data refer to the postintegrative state, together with standard deviations (four transfections each, in addition to two electroporations for pLu and pLu·E, respectively). (B) Analogous experiments for the preintegrative state were obtained by measuring luciferase activities 2 days after transfection. Due to normalizing a number of variables to the expression of a reference plasmid (β-galactosidase, cf. Materials and Methods), the final variations appear smaller.

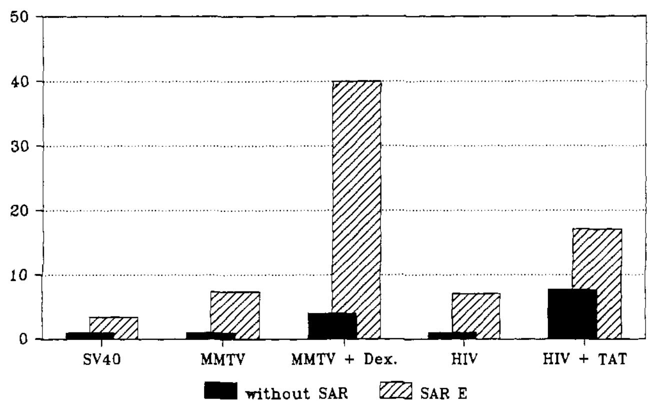

FIGURE 5: A SAR-mediated, enhanced transcription is obtained with a variety of promoters. The indicated promoters were introduced into pLu in place of the 0.43 kb *Hin*dIII fragment. for MMTV, basis activities and activities after dexamethasone treatment are shown; for HIV, the effects were monitored in the presence/absence of a cotransfected RSV-TAT construct. Expression levels for the SAR-free standards in the noninduced/nontransactivated states were arbitrarily set to 1 used for reference. Experiments for HIV/HIV + TAT refer to BHK (baby hamster kidney) cells; all other data were derived from mouse L cells.

of typical SAR sequences. These features explain the apparent cooperation of ATATTTs in mediating SAR functions in vitro and in vivo.

The existence of DNase I hypersensitive regions adjacent to scaffold-attached regions *in vivo* indicates that single-strandedness can also spread into the domain and even involve the transcription unit at times of gene activity (Bode et al., 1986; Mielke et al., 1990). The movement of RNA polymerase through a chromatin domain which is anchored on both sides is thought to give rise to a twin-supercoiled DNA structure with negative supercoiling ("underwound DNA") behind and positive supercoiling ahead of the enzyme (Futcher, 1988). Since underwound DNA is recognized by the scaffold, this may explain previous findings that coding regions become attached to the nuclear scaffold or matrix at times of gene activity [reviewed by Cook (1989) and Fisher (1989)]. It is tempting to ascribe the general enhancing effects (which we observe for SARs linked to various promoters, cf. Figure 5), to these properties.

We have shown in this study that strategically positioned SAR sequences can be utilized to improve the stable expression mediated by integrating vectors. The proposed function of SAR elements to attach genes to the nuclear scaffold is independent of the orientation and partially independent of the distance of these elements (Figure 3). However, some intervening sequences appear to be suited better for the transmission of SAR-mediated structural changes than others (cf. the relative effects of SAR elements upstream and downstream from the marker gene in Figure 4). In the present series of experiments, there is a single situation, where a SAR function is severely impaired, and this is in a position where it is "shielded" by Nm^R sequences in vector site c (Figure 3, constructs pCI_c·C and pCI_c·E). This could be due to highly G+C-rich regions within the Nm^R transcription unit which exhibit silencing functions (Artelt, 1989).

We have also demonstrated that SARs become functional only after the integration of transfected DNA into the genome of the host cell (Figure 4). At this time, a stable DNA–histone complex has formed and a template would require at least one round of replication before its transcription could be reinitiated (Svaren & Chalkley, 1990). However, the establishment and propagation of a state in which a transgene is poised for transcription might be supported by SAR functions [see discussions by Gross and Garrard (1987) and Gasser et al. (1989)]. Prior to integration, a burst of transcription is usually observed ("transient expression") which has been ascribed to the ability of DNA to recruit transcription factors before its final assembly into nucleosomes. At this stage SAR sequences generally have adverse effects (compare the data in panels A and B, Figure 4), probably because attaching a soluble template to the scaffold is disadvantageous. In this context, it should be recalled that one of the functions that have be ascribed to SAR sequences is to recruit topoisomerases which relieve the tension arising during the transcription of an anchored but not of a freely rotating substrate (Adachi et al., 1989).

SAR elements have repeatedly been found to coincide with or to support the function of enhancers (Gasser & Laemmli, 1986; Cockerill & Garrard, 1986, Blasquez et al., 1989; Sperry et al., 1989; Stief et al., 1989; Xu et al., 1989). We noted that SAR elements, selected solely by screening for their scaffold-binding affinity, share certain properies with the classical enhancers such as the distance and orientation independence of their action. Moreover, they require a certain minimum length and a redundancy of sequence information [cf. Schaffner et al. (1988)], and their efficiency is best in the context of a native chromatin structure. The most discriminating feature is the antagonizing action of SAR but not of enhancer elements if they are present on soluble substrates. Other differences concern the proteins which recognize the underlying sequences and particularly the fact that SAR elements usually occur to both sides of a transcriptional unit. This configuration appears to be required for their optimum performance.

Added in Proof

During the preparation of the manuscript, the results of Phi-Van et al. (1990) on the transcriptional effects of SAR sequences from the chicken lysozyme gene were published, expanding the scope of SAR elements which are active with various cellular backgrounds.

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