Scaffold/Matrix-Attached Regions Act upon Transcription in a Context-Dependent Manner[†,‡]

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ABSTRACT: Scaffold/matrix-attached regions (S/MARs) are cis-acting elements with a function outside transcribed regions and in introns. Although they usually augment transcriptional rates, their action is highly context-dependent. We cloned an 800 bp S/MAR element from the upstream border of the human interferon-β domain at various positions within a transcribed region of 4.3 kb. By use of retroviral gene transfer, the vector could be integrated into target cells as a single copy enabling a rigorous definition of the distance between the S/MAR and the transcriptional start site. At a distance of about 4 kb, the S/MAR supported transcriptional initiation, whereas at distances below 2.5 kb, transcription was essentially shut off. Controls proved the functionality of all constructs in the transient expression phase and ruled out any influence of S/MAR position on transcript stability. Moreover, no pausing or premature termination was observed within these elements. We suggest that the protein binding partners of S/MARs change according to the topological status, explaining these divergent S/MAR effects.

It is now generally accepted that the 25 million nucleosomes in a single mammalian nucleus can be organized into about 60 000 chromatin loops, each representing an independent regulatory unit. The domain organization is brought about by the anchorage of certain DNA landmarks, so-called scaffold- or matrix-attached regions (S/MARs),[1] to a skeleton of protein cross-ties called nuclear matrix (interphase) or nuclear scaffold (metaphase; the term is now used in a more general sense). Such landmarks occur at proximal and distal sites flanking the 5′ and 3′ ends of a gene, but also within introns. Examples of single genes divided over two domains are the hamster DHFR gene (Käs & Chasin, 1987), the human topo I gene (Romig et al., 1992), the human interleukin-2 gene (Artelt & Bode, unpublished), a light-inducible gene of plants (Stockhaus et al., 1987; Mielke et al., 1990), and the mouse immunoglobulin κ and μ heavy chain genes (Cockerill & Garrard, 1986; Cockerill et al., 1987). Studies on MPC-11 plasmacytoma cells have shown that 9% of poly(A) mRNA arises from the transcription of the κ locus, indicating that RNA polymerase must initiate transcription every 3.2 s and, if evenly distributed, would occur about every 260 bp along the κ alleles (Cockerill & Garrard, 1986). Since, by necessity, these intronic S/MARs have to be transcribed and since they do not impede passage of RNA polymerase II, their occupation must be regulated.

There is at least one example where a transcribed S/MAR occurs in an intergenic region. This element coincides with the replication origin of the chicken α-globin domain, which is transcribed in normal and transformed erythroblasts as part of a full-domain transcript (de Moura-Gallo et al., 1991). After this transcript has opened the domain in dedicated cells, the S/MAR may reattach to separate the individual transcription units. These observations demonstrate that a S/MAR with a role in S phase can be transcribed, whereas S/MARs coinciding with the domain borders (Targa & Razin, 1994) may define the termini of a replicon [cf. Handeli et al. (1989)].

S/MARs are a relatively novel addition to the class of cis-acting elements. Although they can augment transcript levels, they can clearly be discriminated from enhancers by their inactivity in transient expression experiments (Bode et al., 1995, and references therein). Apart from this now generally accepted criterion, there exist both functional and physical relations. Both elements are composed from certain modules with highly redundant information [sub-S/MAR elements or enhansons, respectively; see Bode et al. (1995), Serfling et al. (1985), Ondek et al. (1988)], and both have a lower critical length of about 200 bp (Kay & Bode, 1995; Schaffner et al., 1988). They frequently cohabit on a certain stretch of DNA and are thought to support each other's function (Boulikas, 1995).

Although enhancers are generally believed to interact with the basal transcription machinery by looping, the actual experimental data supporting such a mechanism are minimal and it has been suggested that the initial contacts made by looping have to be checked by a tracking mechanism to explain the correct choice of a promoter (Weintraub, 1993). Both steps, looping and tracking, may be modulated by the occurrence of S/MARs. Regarding their contribution, there are again divergent views. On one hand, S/MARs may

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[1] Abbreviations: huIFN-β, human interferon-β; IRES, internal ribosome entry site; LTR, long terminal repeat; MAR, matrix-attached region; ORI, origin of replication; PAC, puromycine *N*-acetyltransferase; SAR, scaffold-attached region; S/MAR, consensus term covering SARs and MARs; SEAP, secretory alkaline phosphatase.

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support the targeting of nearby enhancers to the nuclear matrix and provide for their proximity to transcribed units (Boulikas, 1995). Such a mechanism would enable the formation of alternative functional units, activating the respective promoter at the matrix by a directional transfer of transcription factors. On the other hand, following their suggested role as domain borders, S/MARs could limit the action of an enhancer to the domain in which it participates, and this is supported by a number of enhancer-blocking experiments (Bode et al., 1995). Finally, S/MARs might serve the role of a domain opener as first proposed by Zhao et al. (1993). Such a function would be rather universal and would not necessarily require a classification of S/MARs into elements with permanent and facultative attachment properties. A domain-opener function has become attractive by recent findings that changes in chromatin structure caused by a core enhancer only allow for local accessibility while the generation of an extended DNase I sensitivity depends on the simultaneous presence of a S/MAR. Only in this context can an increase in the number of PolII transcripts by 1 order of magnitude be observed (Jenuwein et al., 1993; Forrester et al., 1994).

A fundamental question in understanding S/MARs concerns the mechanism(s) by which these elements operate in eukaryotes to set the topological borders between chromatin domains, to initiate replication, and to augment the transcriptional efficiency of the associated gene(s). Here we demonstrate that a S/MAR that participates in the upstream border of the human interferon-β gene can, in principle, be transcribed in a new, non-natural context. However, the transcriptional properties are highly dependent on the S/MAR−promoter distance, suggesting a complicated interplay with the initiation process.

MATERIALS AND METHODS

Plasmids

The retroviral vector, pM5sepa (called pSP lateron), is a derivative of pM5neo (Laker et al., 1987). It comprises the retroviral sequences necessary for an efficient transcription and packaging, derived from the myeloproliferative sarkoma virus (MPSV). For its construction, the coding sequences were excised from pM5capa (Mielke et al., 1996) and the gap was filled with the aid of linkers, by SEAP (secretory alkaline phosphatase), IRES (internal ribosomal entry site), and PAC (puromycine acetyltransferase) sequences to yield pM5sepa (Mielke, 1993). The 800 bp *Hin*dIII-*Eco*RI S/MAR fragment from the center human IFN-β upstream S/MAR was cloned into several positions, a (*Hin*dIII), b (*Xho*I), c (*Cla*I), and 5′ or 3′ (*Nhe*I), respectively, of pM5SEPA in the same orientation relative to transcription as in the donor domain. For cloning via blunt ends, these sites had to be filled in by Klenow polymerase.

Retrovirus Infection

Transfection and Electroporation. NIH 3T3 murine embryo fibroblasts (ATCC CRL 1658) and ψ-2 packaging cells (Mann et al., 1983) were cultured in DME medium containing 10% fetal calf serum, 20 mM glutamine, 60 μg/mL penicillin, and 100 μg/mL streptomycin and passaged at the time of confluence. Transfection and electroporation routines were as described before (Mielke et al., 1990, 1996). ψ−2 cells transfected with pM5SEPA (derivatives) were supplied with a producer medium (3 mL DME per 25 cm^2 flask). After 24 h, the medium was harvested, filtered through a 0.45 μm membrane, and used for the infection of NIH 3T3 target cells.

Infection. Murine 3T3 target cells were infected by applying conditions that rendered one in one thousand cells resistant. Such a low titer reduces the risk of multiple infections in a given cell (Mielke et al., 1996).

Transfection Protocols

For stable expression experiments, the transfection protocol has been optimized, mainly by adjusting conditions obviating the need for carrier DNA (Klehr et al., 1991, 1992).

For the transient expression experiments in Figure 2B, 3 $\times$ 10^5 NIH 3T3 cells were seeded per 25 cm^2 flask (day 1). On day 2, the respective DNA precipitates were prepared as follows. Three micrograms of the pMSP5 derivative and 2 μg of a luciferase expression standard (pLu; Klehr et al., 1991) were distributed in 200 μL of OptiMEM I (GibcoBRL) and provided with a solution from 12 μL lipofectamin (GibcoBRL) plus 200 μL of OptiMEM I. The mixture was left at room temperature for 40 min, at which time it had turned turbid. Cells were washed with 2 mL of serum-free MEM and subsequently treated with the precipitate plus 1.6 mL of OptiMEM I. At a time when the precipitate became clearly visible on the cells (4 h), 2 mL of DME containing 20% FCS was added. On day 3, cells were washed with 3 mL of DME containing 10% FCS. On day 5, 700 μL of each supernatant was withdrawn for a SEAP colorimetric test. Cells were washed with 2 mL PBS and then harvested in 2 mL of TEN. A 1.5 mL aliquot of the suspension served for the visualization of mRNA on a Northern blot; 450 μL was used for PAC and luciferase tests, and the cell number was determined on the remaining 50 μL. RNA, SEAP, and PAC levels were corrected for variations in transfer efficiency by reference to the activity of the cotransfected luciferase standard (pLu).

Reporter Assays

SEAP was quantified as described by Berger et al. (1988). Use of PAC as a reporter gene followed the procedure of Mielke et al. (1995). Luciferase was tested on cellular extracts prepared by lysing cells in 300−1000 μL of extraction buffer (0.1 M KH_2PO_4, at pH 7.8 and 1 mM DTT). The bioluminescence of 10−50 μL was quantified in a Berthold Biolumat model LB 9500c instrument by integrating the output over the first 10 s and correcting this value for the number of cells in the assay.

Copy Number Determination

Copy numbers were quantified by a refined Southern analysis using two probes, one for an intrinsic gene element (murine IFN-β promoter) as the internal control and the IRES−PAC segment from pM5sepa. The sequences of both probes were cloned into a single vector (pIP, see Figure 1B). Restriction of this vector with the enzymes used for genomic DNAs and its application for Southern analyses grants the presence of both probes at a stoichiometric ratio on each filter. Quantification of autoradiographs was achieved with a Molecular Dynamics phosphoimaging system.

Purification of Genomic DNA

Cells from a 75 cm^2 culture flask were processed as described (Mielke et al., 1996).

Northern Blots and Internal Controls

Expression levels for the dicistronic transcript were determined by the probes indicated in Figure 2 or 5 and referenced to the respective signal arising from endogenous murine pyruvate kinase (PK) gene transcription. In detail, cells from a 75 cm^2 culture dish were washed twice with PBS followed by TEN. The pellet was resupended in 2 mL of Trizol (BRL) and then treated according to the manufacturer)s protocol.

Messenger RNAs. RNA (300 μg) was either used directly (Figure 5) or enriched for oligo(A) RNA by the Dynabeads procedure (Dynal Inc.). Controls show that this procedure, besides oligo(A) RNA, enriches for S/MAR containing messages and does not exclude any particular type of RNA.

Electrophoresis and Blotting. Oligo(A) mRNA (3 μg) or total RNA (10 μg) was dried and dissolved in a mixture of 2 μL of H_2O, 5 μL of deionized formamide, 2 μL of deionized formaldehyde, 1 μL of 0.2 M MOPS/1 mM EDTA/10 mM NaOAc (pH 7), 1 μL of ethidium bromide (400 μg/mL), and 1 μL of 0.2% xylene cyanol/0.2% bromophenol blue/10 mM EDTA (in 50% glycerol). The sample size was sufficient for two trays of an 1.2% agarose gel containing 6.6% formaldehyde. Electrophoresis proceeded for 3−5 h in 20 mM MOPS/1 mM EDTA/1 mM NaOAc. The gel was successively washed in water (1x) and 10x SSC (2x) and blotted onto GeneScreenPlus (Dupont) with 10x SSC.

Hybridization. The blot was subjected to a prehybridization step (2 h, 60 °C) and the final hybridization in a volume as small as possible (24 h, 60 °C). Hybridization solution (40 μL) contained 2 mL of 20% SDS, 4 g of dextran sulfate, 2.32 g NaCl, and 35.5 mL of H_2O. These components were dissolved at 60 °C and provided with 2.6 mL of a mixture composed from the following aqueous competitior solutions: 1.3 mL of herring sperm DNA (10 mg/mL) and 1.3 mL of yeast tRNA (10 mg/mL), denatured at 95 °C for 15 min. After hybridization, several washes were applied to remove excess probe: 2x SSC (2 × 5 min at room temperature), 2x SSC containing 1% SDS (1 h, 65 °C), 2x SSC (3 × 5 min at room temperature), and 0.1x SSC (3 × 5 min at room temperature). In case of a high background, the procedure could be concluded with an additional wash step at higher stringency (0.1x SSC, 1% SDS, and 65 °C).

Run-on Transcription

Preparation of Nuclei. NIH 3T3 cells were collected from a confluent monolayer by centrifugation (800*g*, 5 min), washed with PBS, and then treated according to the protocol of Groudine et al. (1981). The final nuclear pellet was suspended in nuclear freezing buffer (40% glycerol, 50 mM Tris/HCl, at pH 8.3, and 5 mM $MgCl_2$) and frozen in liquid nitrogen as 100 μL aliquots containing 4×10^6 nuclei each.

Run-on reaction. A 100 μL aliquot of nuclei was provided with 30 μL of 5x run-on buffer (25 mM Tris/HCl at pH 8, 12.5 mM $MgCl_2$, 750 mM KCl, and 1.25 mM each of ATP, GTP, and CTP), 10 μL of [α-^{32}P]UTP (3000 Ci/mmol), and either 0.06% (low) or 0.6% (high) Sarkosyl (SERVA). Sarkosyl concentrations were adjusted by adding 10 μL of a 15x stock solution with either 0.9 or 9% detergent. The mixture was incubated for 30 min at 30 °C. The reaction mixture (150 μL) was then transferred into an Eppendorf cup containing 1.5 mL of Trizol (BRL) and shaken at 1000 rpm until a homogeneous suspension resulted (2 min). $CHCl_3$ (300 μL) was added, and shaking was continued for an additional 5 min. After centrifugation (12000*g*, 15 min, 4 °C), the clear aqueous upper phase was transferred to a fresh Eppendorf cup, provided with 800 μL of 2-isopropanol, and shaken for 5 min at room temperature. Following an analogous centrifugation step (12000*g*, 15 min, 4 °C), the supernatant was withdrawn and then washed twice with 1 mL of 75% ethanol at −20 °C. After briefly drying, the pellets were dissolved in 100 μL of water at 60 °C and kept on ice. Incorporation was determined via Cerenkov counting on a 1 μL aliquot (typically $1-2 \times 10^5$ cpm). RNA was either used directly for hybridization or stored at −70 °C until use.

Blotting. For Figure 4A, restriction fragments were applied to a slot blot apparatus (Millipore) at a minimum of 100 ng per slot. The transfer of DNA to Gene Screen Plus (DuPont) was performed in 0.4 M NaOH. The membrane was then neutralized by soaking in 2x SSC and heated to 80 °C in a thermo oven (2 h). For Figure 4B, fragments were separated electrophoretically on an agarose gel and then blotted to Gene Screen Plus. The hybridization steps were essentially as above but included the following modifications. The 2.6 mL competitor solution contained 500 units of RNAsin (Promega) and 40 mM DTT in addition, and the hybridization period was extended to 42 h. (ii) The washes included a step with 2x SSC and 10 μg/mL RNase A (10 min at 37 °C) between the washes with 2x SSC at room temperature. Where applicable, the integrity of RNA was checked on a sequencing gel by applying 10^5 cpm which should produce a smear up to a 5 kb marker.

RESULTS

The retroviral vector pM5SEPA (called pSP for brevity) was first developed to recover sites of retroviral integration and to quantify their transcriptional potential (Mielke et al., 1996). For this purpose, it comes with a selectable marker (PAC) and a reporter gene (SEAP) which are both driven by the viral promoter/enhancer functions in the 5-LTR; polyadenylation is mediated by the 3′-LTR. A sensitive reporter assay was developed for the PAC gene whereby the coexpression and integrity of both cistrons could be established (Mielke et al., 1995). A systematic comparison with a first-generation retroviral vector (pM5CAPA) in which the selectable marker and the reporter were driven by separate promoters showed that only the bicistronic constructs prevented unpredictable regulatory phenomena which are commonly ascribed to promoter suppression (Mielke, 1993; Mielke et al., 1996).

The possibility of transferring intact single copies into a recipient cell by retroviral functions and the availability of an extended (4.3 kb), functional dicistronic expression unit provides an ideal system to investigate the influence of S/MAR elements, cloned into various positions along the transcribed region (Figure 1A). Positions a−c flank the SEAP and PAC genes, respectively. Position 3′ marks the *Nhe*I site of the 3′-LTR which accepts considerable inserts with little or no functional interference (Stuhlmann et al., 1989). During reverse transcription of the retroviral RNA genome, the 3′-LTR serves as a template for 5′-LTR synthesis. Therefore, pSP-3′, if transferred by infection, consistently yields a pS-5′,3′-provirus (Figure 1A). Transfection on the other hand requires the construction of the complete double-S/MAR vector (pSP-5′3′) but enables separate experiments on the corresponding single-S/MAR

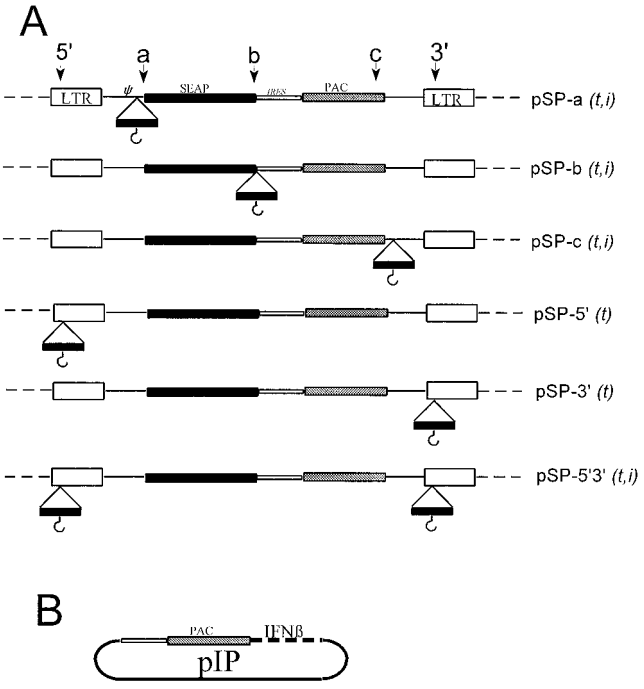

FIGURE 1: Constructs. (A) Structure of pM5sepa-S/MAR (pSP-*n*) integrants which can be obtained by transfection (t) or infection (i). *n* refers to the location of the 800 bp S/MAR insert. (B) pIP, plasmid used for determining the number of integrated copies for pM5SEPA derivatives by reference to the endogenous murine interferon-β (IFN) gene.

constructs (pSP-5′ and pSP-3′) in addition. The S/MAR element chosen was derived from the putative human interferon-β (huIFN-β) upstream chromatin border. The in vitro and in vivo properties of this 800 bp element [called IV in Mielke et al. (1990)], which has become one of our prominent S/MAR standards (SAR_{800}), have been extensively studied (Mielke et al., 1990; Kay & Bode, 1994, 1995; Dietz et al., 1995). It comprises a pronounced base-unpairing region which has been implicated in central S/MAR functions (Bode et al., 1992, 1996).

As a first functional test, pSP or its derivatives were transferred into NIH 3T3 cells by lipofection and the expression was monitored during the transient phase (Figure 2B). This experiment reveals for all S/MAR constructs a consistent, moderate transcriptional down-regulation prior to integration which is not uncommon at this phase at which a final, ordered chromatin structure is still lacking (Klehr et al., 1991). This down-regulation is evident from mRNA levels (solid bars) which in most cases are paralleled by the activities of the reporter enzymes [see Mielke et al. (1995)]. An obvious exception arises for the SEAP levels monitored for the pSP-a vector which is a consequence of the direct apposition of the S/MAR and the SEAP start codon as the S/MAR provides as many as 15 AUGs in front of the actual one. It is therefore concluded that, with the exception mentioned, there is no major interference arising from the stability of individual mRNAs or major translational contributions to the expression levels.

Next, the analogous assays were performed for integrated copies of these constructs after several cell divisions. At this stage, all transgenes are presumed to be organized as chromatin and it is thought that S/MAR elements for which

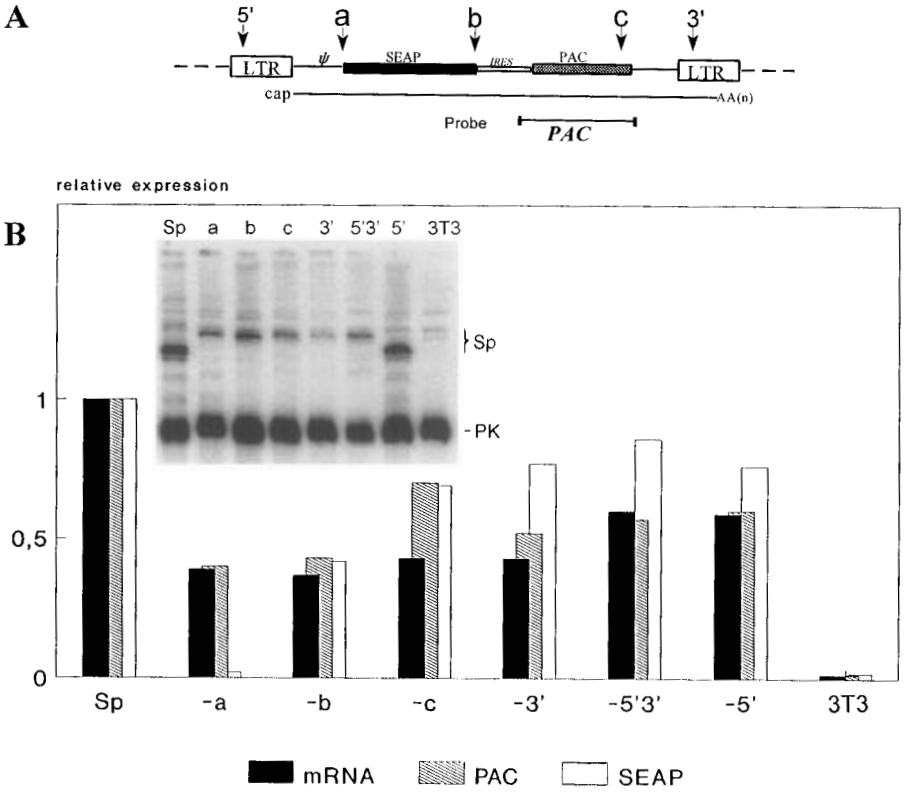

FIGURE 2: Structure and performance of pSP derivatives in the transient state. (A) The localization of the 800 bp S/MAR fragment in either position a, b, or c in the 3′-LTR (3′) or in the 3′- and 5′-LTRs (3′5′) of pSP derivatives is shown as is the location of the bicistronic transcript and the 1.2 kb PAC probe used for the Northern analyses in part B. (B) Enzyme activities of PAC and SEAP have been determined as mentioned in Materials and Methods. The level of the bicistronic transcript has been derived from Northern blots (example shown in the inset), using the 1.2 kb PAC probe, together with a pyruvate kinase (PK) probe. pSP transcripts (Sp) correspond to either of two sizes depending on the S/MAR insertion site. Expression levels were corrected for variations in transfer efficiency by reference to the activity of a cotransfected luciferase standard (pLu).

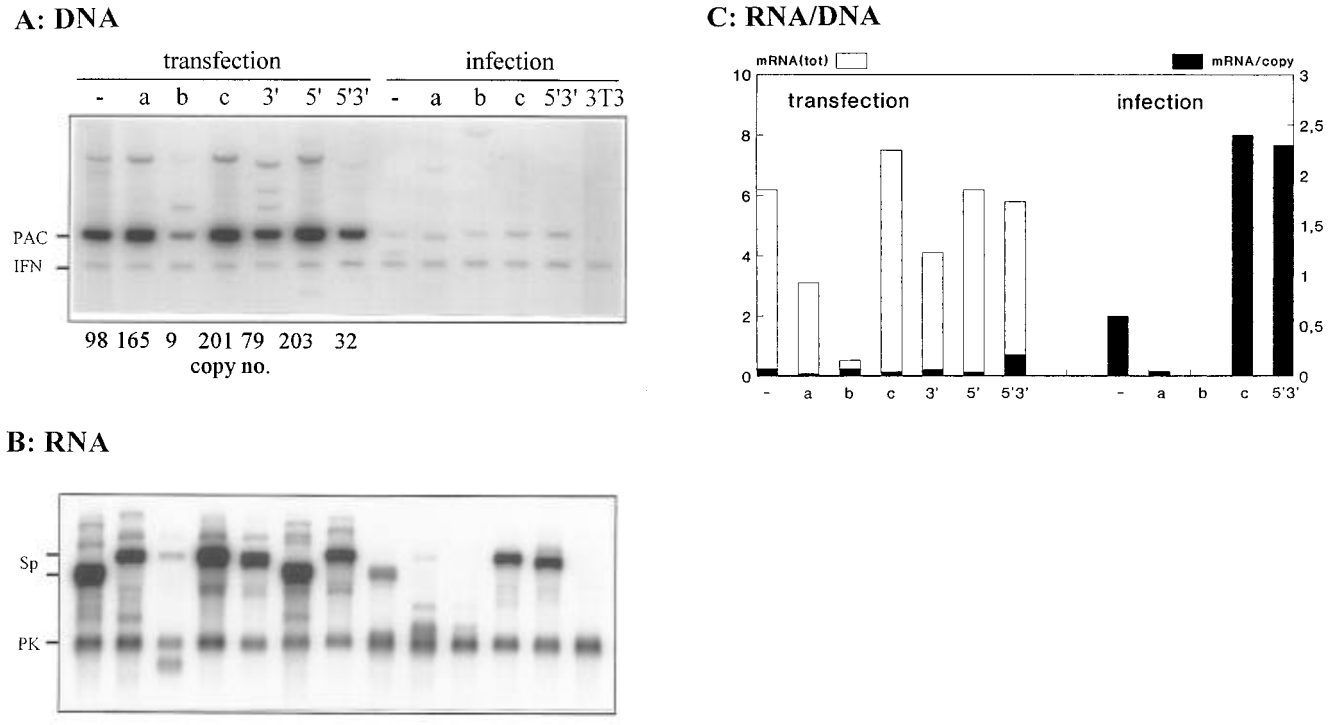

FIGURE 3: Copy numbers and (stable) expression levels for the pSP derivatives of Figure 2A after integration. (A) Copy numbers for vectors transferred by transfection or retroviral infection have been determined from a Southern blot using pIP (Figure 1B) as a probe. The rightmost trace refers to empty (nonmanipulated) NIH 3T3 cells. (B) Northern blots characterizing expression of the vectors in part A. The probe is the one indicated in Figure 2A. (C) Bar diagram reflecting the expression levels before (open bars) or after correction for copy numbers (solid bars).

positioned nucleosomes are apparently lacking (Bode et al., 1995) are engaged in interactions with the limited number of binding sites found in the nuclear matrix or nuclear scaffold (Kay & Bode, 1994). Gene transfer was performed either by transfection which tends to yield tandem arrays (concatenates) of the transgene, including vector sequences, or by retroviral infection which, at limited MOI (multiplicity of infection) levels, is easily performed in a way yielding a single copy of the construct. In the latter case, the insert ends at the retroviral LTRs. Except for an integration-associated duplication of a short stretch of host sequences (in this case four base pairs), there is no further rearrangement and the insert is free from vector sequences. Copy numbers were established by a refined Southern blot procedure based on a composite probe (pIP, Figure 1B) which generates a simultaneous signal for an internal standard derived from murine IFN-β. It is clearly seen that the infection route generated the expected series of single-copy integration events (Figure 3A). For transfection on the other hand, copy numbers varied more than 20-fold. Even more important, the copy number appeared to correlate with the respective construct as it could be reproduced in several separate experiments using various batches of DNA. It will be shown later that these numbers roughly parallel expression levels and this phenomenon appears to arise from the instability of loci harboring multiple identical copies of DNA.

Regarding the steady state level of mRNA, data from the retroviral infection were directly interpretable (Figure 3B) and they revealed a very pronounced S/MAR position-dependent modulation that was not found in the transient expression series shown in Figure 2B.

(i) At the promoter proximal positions, 580 and 2530 bp downstream from the cap site (pSP-a and pSP-b), the S/MAR caused a near-complete suppression of transcription.

(ii) At the promoter distal positions, 4020 bp downstream from the cap site (pSP-c) and also in the case of the double-S/MAR construct pSP-5′3′ (one S/MAR ending at position - 400 and the other starting at +4200), S/MAR(s) exerted a 4-fold increase of transcription relative to the control.

In contrast, the data arising from transfection are highly ambiguous, and considering the vast variation of copy numbers, it appears doubtful that reference to a single copy is permitted for a comparison of expression levels. While the raw data (Figure 3C, light bars) seem to reproduce some aspects of the infection series, this marked profile is lost after calculating the per-copy expression. Generally, the size of the dominant transcript conforms to expectations, i.e. reflects lengths of 4.3 kb for the S/MAR-free control and in case a S/MAR is located upstream from the cap site (pSP-5′). It covers 5.1 kb if the S/MAR is part of the transcribed cassette (Figure 3B). In addition, the transfection series produces a number of longer transcripts as well which is thought to reflect incomplete termination within the 3′- LTR and which is typical of a tandem array of genes. For the reasons mentioned above, integrants of the type pSP-5′ and pSP-3′ can only arise from transfectio; a retrovirus-mediated transfer of pSP-5′ would result in the apparent loss of the 5′-S/MAR, while a transfer of pSP-3′ would create a pSP-5′3′-type provirus.

Summarizing so far, we are left with a situation where S/MARs exert a highly context-dependent influence on transcription which is linked to the integrated state of the transgenes. Therefore, it is likely that we have to deal with a genuine S/MAR function (Bode et al., 1995), the molecular basis of which has to be established. Differences in mRNA stability were unlikely to contribute as shown by the similar mRNA levels for pSP-a, pSP-b, and pSP-c in the transient test (Figure 2B) compared to the all-or-none effect found

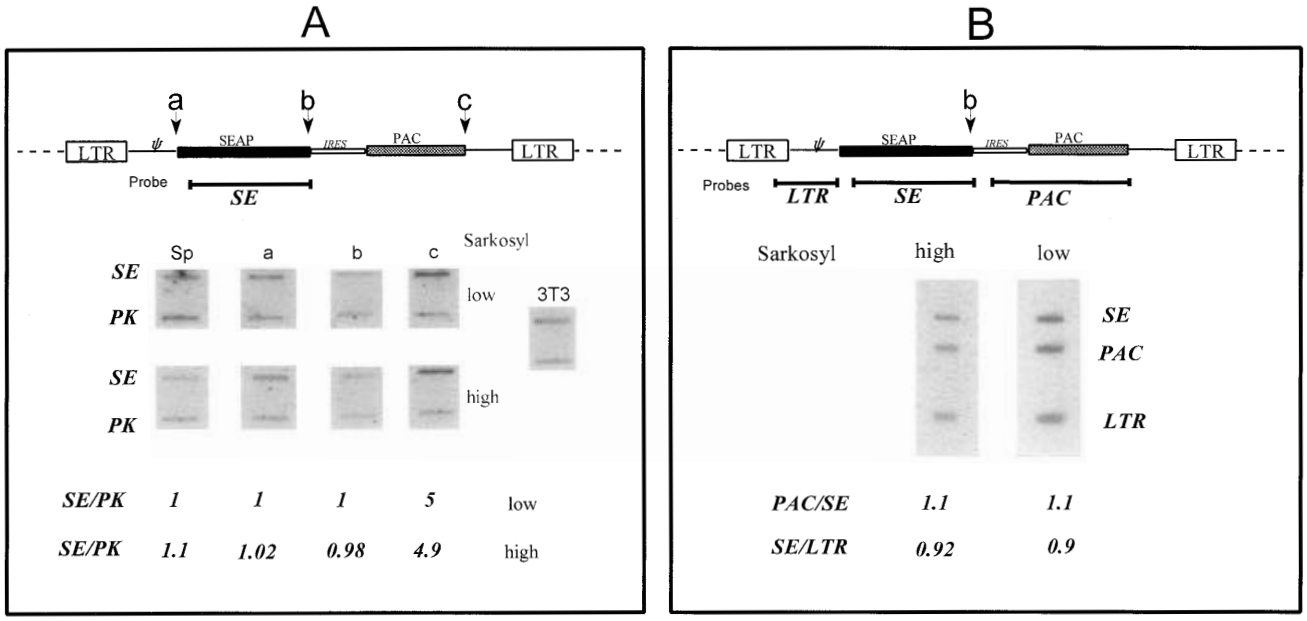

FIGURE 4: Molecular basis of S/MAR activity: run-on transcription assays. (A) SEAP (SE) and pyruvate kinase (PK) DNA fragments on a slot blot were labeled by hybridization with run-on transcripts. These transcripts were generated from NIH 3T3 cells infected with pSP constructs harboring the S/MAR element in positions a−c and from a S/MAR-free control (Sp). An analogous probe was also isolated from empty, i.e. uninfected, cells (3T3). (B) Three fragments of Sp-b, obtained by restriction, were separated electrophoretically. A blot of the gel was labeled using radioactive run-on transcripts from 3T3 cells expressing transfected copies of this construct. Although application of high concentrations of Sarkosyl (0.6%) yields an about 3-fold acceleration of transcription rates (not shown), this cannot be ascribed to a disruption of S/MAR−protein iteractions as the signal ratios PAC/SE and SE/LTR remain constant.

for the corresponding constructs in the infection series (Figure 3B). We are aware of one study which demonstrates that S/MARs act by supporting transcriptional initiation (Phi-Van et al., 1990), but in that case, the S/MARs were found in a more classical position outside the transcribed region of a transfected and thereby probably multimeric expression cassette. While a position-dependent negative influence upon transcriptional initiation appeared to be conceivable, pausing or premature termination could also be easily reconciled with current models of S/MAR function.

Nuclear run-on transcription assays are a common tool for monitoring the distribution of transcriptionally active RNA polymerase molecules; after nuclei are pulse-labeled with radioactive nucleoside triphosphates, the synthetized RNAs are used to probe defined restriction fragments for ongoing transcription on their counterparts in the nucleus. Usually, these tests are done at concentrations of an anionic detergent, Sarkosyl, just high enough to prevent re-initiation of polymerase. The strength of resulting signals is then taken as a measure of the relative density of transcribing polymerases along the transcription unit. In addition, these assays can be performed in the presence of higher Sarkosyl concentrations which will strip histones and the vast majority of non-histone proteins. In the presence of up to 2% Sarkosyl, RNA polymerase II that has initiated transcription but has been paused by an impediment will resume transcript elongation once these conditions are adjusted (Gariglio et al., 1981; Rougvie & Lis, 1990).

For Figure 4A, a SEAP fragment (SE) and, as an internal control, a pyruvate kinase fragment (PK) were spotted on slot blots which were subsequently probed by radioactively labeled RNAs isolated from cells harboring the parent construct pSP (pM5SEPA) or the S/MAR derivatives pSP-a, pSP-b, or pSP-c. Parallel experiments were run in the presence of low (0.06%) and high (0.6%) concentrations of Sarkosyl. A critical evaluation of these experiments leads to the following conclusions.

There is a factor of 5 increase of signal strength for pSP-c relative to that of the control (pSP). Due to a background in the SEAP signal (see below), this is a minimum estimate proving that an appropriately positioned S/MAR, even if located within the expression cassette, can augment transcriptional initiation to a considerable extent. To our knowledge, this is the first demonstration of this property at the single-copy level, i.e. under conditions eliminating any composite effects from the multiple S/MARs of a concatenate produced by transfection techniques.

For construct pSP-a, high concentrations of Sarkosyl do not visibly increase the polymerase density within the probed fragment (SE); therefore, these data provide no evidence for polymerases paused at a S/MAR in position a.

The down-regulation for pSP-a and pSP-b that is evident from Figure 3 is not apparent in this experiment. However, this is obviously due to the limited signal strength obtained from a single integrated copy; an additional test showed that all cells, even the nonmanipulated ones (3T3), give rise to a background signal for the SEAP probe, most likely due to endogenous phosphatase transcripts (Figure 4A, right-hand pannel). Since such a background presents no problem if higher transcriptional levels are enforced by multiple copies (see below), we have obviously approached the detection limit of the system.

In order to rule out any obvious effect of S/MAR sequences on transcript termination or elongation, an application of conventional transfection techniques appeared justified since the outcome of *these* experiments should be unaffected by the route of gene transfer. For polyadenylation, at least two cis-acting regulatory signals, a highly conserved consensus AAUAAA sequence (or a variant AGUAAA) 10−30 nucleotides upstream of the polyadenylation site and a weakly conserved G+U or U-rich sequence 1−30 nucleotides downstream of the poly(a) site, are required. Premature termination within S/MARs could occur due to the occurrence of AATAAA and AGTAAA tracts

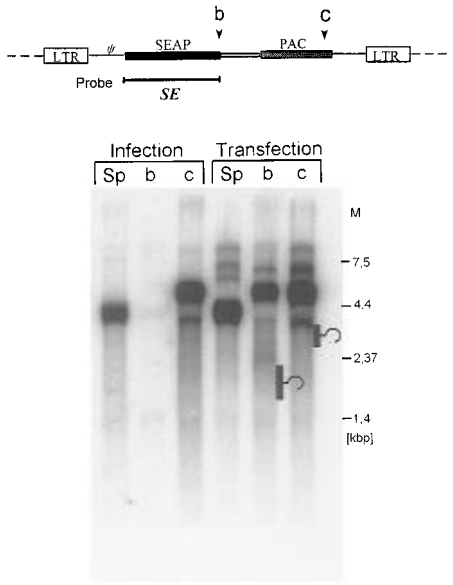

FIGURE 5: Transcription through S/MARs: Northern blots. Total RNA was isolated from NIH 3T3 cells that have obtained Sp-b or Sp-c by either transfection or infection. Northern blots were generated using a proximal probe (SE) which would reveal premature termination (or pausing) within S/MARs (see S/MAR symbols next to traces marked b and c).

which are present in SAR_{800}. Moreover, pausing for RNA polymerase II has been found at sites where the downstream sequence includes runs of phased dA:dT tracts of the type forming bends in DNA (Kerppola & Cane, 1990). Since bending is one of the common features associated with S/MARs (von Kries et al., 1990), such an effect had to be excluded. This has again been done by a run-on assay (Figure 4B) and by visualizing transcripts on a Northern blot (Figure 5).

The run-on test was performed on construct pSP-b which enables probing before and behind the S/MAR element to monitor any discontinuity of polymerase distribution. While the signals for the S/MAR and PAC were entirely specific, i.e. not found by hybridizing with labeled run-on transcripts from nontransfected 3T3 cells, there was again a very weak signal due to endogenous phosphatase transcripts (not shown). Since this background was below 5% of the authentic signal generated from the transfected SEAP construct, it presents no complication. For the analysis in Figure 4B, high Sarkosyl concentrations increased the general signal strength about 3-fold. Since there are several examples of genes for which the transcript elongation is unaffected by Sarkosyl, this may indicate the presence/disruption of nucleosomes. On the other hand, no differences in the relative occupancy of fragments could be monitored under low- and high-Sarkosyl conditions, respectively (see the PAC/SE and SE/LTR ratios in Figure 4B), and this again provides no evidence for a retardation of polymerases mediated by proteins that interact with the S/MAR sequence.

A final experiment designed to rule out any contribution of prematurely terminated or paused mRNAs to the transcriptional influences mediated by S/MARs was a Northern blot on the total RNA of cells harboring pSP-b or pSP-c. The promoter proximal probe used (marked SE in Figure 5), in contrast to the PAC probe in Figure 3, would reveal any mRNAs ending within the S/MAR elements at positions b or c. In case of infection-mediated transfer, strong signals for full-length mRNA were only produced by the control (pSP) and by constructs with a S/MAR in a stimulatory position (pSP-c). For pSP-b, there were barely any signals, neither for the full-length nor for partial transcripts. For the transfection series, pSP-b transcripts became clearly visible, but again, there is no indication for partial transcripts ending within the S/MAR. It is therefore suggested that both positive and negative regulation occur at the level of transcriptional initiation, and any model explaining these actions of S/MARs should take this conclusion into account.

DISCUSSION

S/MARs Constitute a Special Class of Cis-Acting Elements. The number of binding sites on the nuclear matrix or scaffold for S/MARs is limited [30 000 for the linearized, double-stranded form of SAR_{800}; see Kay and Bode (1994)] and is inversely related to S/MAR length, demonstrating a composite mode of attachment. The matrix also accommodates negatively and positively supercoiled as well as single-stranded DNA (Bode et al., 1995, 1996). Among these, the recognition of single strands is one of the probably several mechanisms by which S/MARs are recognized in the living cell. This is a consequence of their content of homooligonucleotide stretches and inverted and direct repeats which account for the presence of base-unpairing regions (BURs) due to the formation of triple-helical DNA, cruciforms, and slippage structures which have in common the exposure of single strands (Bode et al., 1995; Mielke et al., 1996). After transfection, the activity of S/MARs can only be traced at a time when a definite chromatin structure has been attained during one or several rounds of replication. This applies to transgenes that have integrated at low copy numbers and also to episomally replicating vectors [Hörtnagel et al., 1995; review in Bode et al. (1995)]. In contrast, S/MARs behave neutrally or even exert negative actions in any transient assay.

It is obvious that the proteins constituting the nuclear matrix must have diverse recognition modes for DNA structures as these proteins have been implicated in such diverse processes as transcription, RNA processing, replication, and DNA repair. While stable contacts between S/MARs and their binding partners have prevailed in early models of S/MAR function, recent data point at a more dynamic relationship. It has been known for some time that the matrix S/MAR contacts of cut and thereby topologically unrestrained DNA loops are accessible to S/MAR competitors and that S/MARs can be electroeluted from embedded, permeabilized cells after a treatment with restriction enzymes (Eggert & Jack, 1991; Hempel & Strätling, 1996). Superficially, these findings seem to indicate that for these S/MARs there is no firm matrix contact in vivo. However, for all these investigations, by necessity, the topological state had to be perturbed by restriction. A more reassuring approach is therefore based on the reversible cisplatin cross-linking technology developed by Ferraro et al. (1992, 1995). Treatment of living cells with the reagent and a subsequent identification of the DNAs and proteins participating in cross-linked complexes prove the participation of authentic S/MARs and matrix proteins for differentiated tissues from chicken (Ferraro et al., 1996).

Special Elements Require Specialized Techniques for Their Investigation. Classical gene transfer techniques such as transfection or (to a limited extent) electroporation consistently lead to the integration of multiple copies in the form of tandem arrays. It is sometimes ignored that this status may invalidate any conclusion about the distance-dependent

action of a S/MAR upon a promoter since these elements occur at various distances within the transgene complex. An additional complication may arise from the finding that certain S/MAR constructs tend to integrate at higher copy numbers relative to the S/MAR-free controls reflecting the high recombinogenic potential of these sequences (Bode et al., 1996). Due to a lack of sufficient appropriately spaced sites on the matrix, only a limited number of these elements may be accommodated, leading to a higly undefined cluster of functionally different gene copies. Even more serious is the fact that the expression of increasing copy numbers may not lead to higher expression levels, a phenomenon that is pronounced in the plant field where it has been termed homology-dependent gene silencing or cosuppression (Spiker & William, 1996). Similar complications are not uncommon in animals where very high copy numbers provoke unexpected interactions and cellular defense mechanisms like methylation/mutagenesis and excision leading to a transcriptional shutoff for most members of the insert (Kricker et al., 1992; Dorer & Henikoff, 1994). An illustrative example is found in a recent reinvestigation by Kalos and Fournier (1995) of earlier results from transgenes flanked by the apolipoprotein B S/MARs. S/MAR-specific insulator functions which were clearly present at the single-copy level became blurred for multiple-copy integration events. In principle, these limitations can be overcome by the use of retroviral vectors which, when transferred by the infection route, give rise to the integration of one intact copy devoid of plasmid sequences (Mielke, 1993).

Limitations Imposed by the Necessity of a Selectable Marker. Both transfection and retroviral infection techniques involve relatively rare events which require, for their enrichment, the use of a selectable marker. Since this marker has to be expressed at a threshold level, a selection bias is created against low producers. In the present series of experiments, two phenomena occurred which are interpreted as being consequences of selection pressure.

In the transfection experiments of Figure 3 and all subsequent controls, it was established that the lowest overall producer, pSP-b, resulted in an unusually low number of integrated copies. We hypothesize that, due to a cosuppression-type effect, an expression threshold can only be reached in the case where a low-copy number transgene has integrated into a favorable chromosomal site. In contrast, high-copy number arrays, which may lead to high initial expression levels, will deteriorate with time and hence be continuously removed by selection. In the case of the survivors, a few copies profit from the transcription-promoting potential of host sequences leading to the paradoxon that the selected clones harboring pSP-b exhibit a rather normal per-copy expression in the transfection series.

It is hard to conceive how the selection pressure can be met for pSP-b after infection-mediated transfer, i.e. in a situation where virtually no transcripts are detected. Using our reporter gene assays, we found that, for the infection series, SEAP activity closely followed the evaluation of the Northern blot while PAC in all cases, even for pSP-b, yielded a threshold amount of activity. We could ascribe this, in part, to an enrichment of rare deletion events eliminating SEAP with the maintenance of PAC. As such deletions are never found in the transient expression series (Figure 2B), they do not reflect an intrinsic instability of the transcribed sequences. They are rather the result of an extraordinarily low transcriptional potential of the mother construct which enforces the selection of variants that express PAC at a minimum level by processes involving deletion or deregulation.

S/MAR Actions upon Transcriptional Initiation. In a natural context, S/MARs do occur either on the putative boundaries of a chromatin domain, in close association with an enhancer [cohabitation phenomenon, see Gasser and Laemmli (1986)], or in intronic positions. Clearly, in these examples, there are S/MAR sequences which are transcribed and others that are not. Therefore, it has frequently been assumed that S/MARs fall into different classes [review in Boulikas et al. (1995)], and it has been postulated that the attachment of the transcribed class has to be regulated.

For the present study, we have chosen a high-affinity fragment (SAR_{800}) from a S/MAR that is thought to define the upstream boundary of the human interferon-β gene (Bode et al., 1995). Our data show that even this sequence can be transcribed in principle, and it does not cause pausing or premature termination which might have been expected on the basis of certain structural or sequence features that are typical of these elements. Even more important, transcribed S/MARs, if positioned appropriately, can substantially support expression levels. Two parameters have been considered to explain distance effects for S/MARs upon transcription. First of all, the size of the S/MAR-generated loop may be important for S/MAR efficacy. Data from *Drosophila* genes have suggested an inverse relationship between loop size and transcription between 5 kb (histone gene cluster) and about 100 kb (Gasser & Laemmli, 1987). Second, the promoter−S/MAR distance could be an important parameter since the affinity of the S/MAR for the nuclear matrix may force a nearby promoter to be too close to the matrix for optimal functioning (Mlynarova et al., 1995).

Our selection of constructs contains a genuine minidomain (pSP-5′3′), obtained either by cloning SAR_{800} into the *Nhe*I site of both the 3′- and 5′-LTRs (transfection series) or, more elegantly, by using the retroviral duplication mechanism which generates a pSP-5′3′ provirus if a pSP-3′ construct has been packaged into an infectious virus particle (see Figure 1A). In this minidomain, the S/MARs are about 4.5 kb apart, but while the simultaneous presence of two elements augments transcriptional levels about 4-fold, there is no improvement relative to our best single-S/MAR construct (pSP-c in Figure 3, infection series). This could mean that in the given cell type the domain size is near the lower limit which still permits the simultaneous attachment of both S/MARs or that the apposition of S/MARs to important regulatory functions does not allow an improved initiation or termination.

Our data suggest that, for optimum performance, a S/MAR element has to occur at a certain minimum distance downstream from the promoter (5′-LTR), and run-on analyses demonstrate that transcriptional augmentation is effective at the level of initiation (Figure 4A). Since this conclusion could be drawn for a single integrated copy devoid of any vector sequences, it is the first demonstration of a positive transcriptional S/MAR effect at a strictly defined distance between this element and the promoter. In striking contrast, S/MARs cause a nearly complete down-regulation at promoter proximal positions (pSP-a and pSP-b). Since this phenomenon cannot be explained by a reduced mRNA stability, polymerase pausing, or premature termination, initiation is probably again the level that is affected. Like other S/MAR-related actions, this sharp modulation of the transcriptional state is limited to the stable state; in transient

experiments, there is nothing but a uniform, slightly negative S/MAR effect and no difference regarding mRNA levels between the constructs which show the the most divergent characteristics after integration (pSP-b and pSP-c).

A Model. S/MARs are sometimes found in close association with enhancers, but never with promoters. Therefore, a straightforward explanation for the transcriptional shutoff in constructs pSP-a and pSP-b (Figure 3) would be sterical interference of a close-by attachment site with the formation of an initiation complex. However, this simplistic view would not necessarily account for the observation that a S/MAR in position 5′ does not appear to affect initiation to the same extent or for the finding that a S/MAR in an alternative position is apparently transcribed with ease. Therefore, we propose a dynamic model which is based on recent evidence that the binding partners can change as a function of the cell type and transcriptional status (Ferraro, 1995; Bode, 1995).

Like enhancers, S/MARs contain sequences that would be appropriate for the binding of a multitude of transcription factors. More generally, they seem to be associated with proteins whose sequence requirements are rather relaxed (Boulikas, 1995). Low-affinity binding of individual molecules does hence result in specific protein−protein interactions on DNA which lend themselves to serve specific regulatory roles (mass binding phenomenon; Zuckerkandel & Villet, 1988). The ubiquitous members of this protein class can explain the binding characteristics of a total scaffold. Histone H1 interacts with S/MAR DNA in its double-helical state via the minor groove, inactivating a chromatin domain. Triggered by another minor groove binder, HMGI/Y, H1 can be displaced in a cooperative fashion as a prelude to transcription (Laemmli et al., 1992), and subsequently, binding may be taken over by single-strand-specific proteins which will prevent the rebinding of H1 (Ludérus et al., 1994). Abundant members of this class are found among the intermediary-filament-type hRNP proteins which can even accommodate both single-stranded DNA and double-stranded S/MAR DNA, the latter via minor groove contacts (Mattern et al., 1996).

The most plausible model of the transcriptional process involves reeling the DNA template through the polymerase which is a rather immobile part of the nuclear matrix. During this process, the template becomes overwound ahead of the polymerase and underwound behind it (Wu et al., 1988; Cook, 1989). While overwinding is stabilized in the form of positive supercoils made up of B-type DNA, underwinding can lead to either plectonemic (negatively supercoiled) or paranemic (unwound, i.e. base-unpaired) structures. Due to their unwinding potential (Bode et al., 1992), the presence of S/MARs would shift the equilibrium in favor of the unwound form which could be stabilized by the S/MAR binders mentioned. Supercoils can also be accommodated on the nuclear matrix, but on sites not overlapping the ones specific for S/MARs (Kay & Bode, 1994). Among the matrix components stabilizing supercoils are HMG proteins -1 and −2. Supercoils and supercoiling-induced structures like crossovers (Zechiedrich et al., 1990, Corbett et al., 1992; Roca & Wang, 1992) and hairpins (Froelich-Ammon et al., 1994) are also the dominant features that are recognized and converted by topoisomerase II (Osheroff et al., 1991; Roca et al., 1993; Wang & Dröge, 1996).

The outcome of our experiments can be interpreted within this conceptual framework. The default state of S/MAR elements in the poised state, i.e. in a domain that is transcriptionally competent but not actively transcribed (Gross & Garrard, 1987), is single-stranded (Paul & Ferl, 1993; Targa et al., 1994; Bode et al., 1996). This state is probably constrained by the binding of single-strand-specific proteins. The mode of binding will change during phases of gene activity, and this is supported by experimental evidence using enzymatic as well as chemical probes to determine the S/MAR status in living cells or nuclei (Ferraro et al., 1996; Bode et al., 1995). As long as the strands of S/MAR DNAs are held separately to prevent rotation of the helix about its own axis, the positive supercoils arising ahead of an approaching polymerase cannot pass the attachment point. This could be the situation in the case of pSP-a and pSP-b where the binding mode cannot be switched from ssDNA to dsDNA and transcription is stalled. The question is how such a switch could be operated, i.e. what causes the transfer of S/MAR DNA to alternative binding sites which are located on the same or another set of proteins.

We suggest that the first polymerase to transcribe has to build a threshold of positive superhelicity to bring about these changes, and this is possible only if the S/MARs are located at a certain distance. Moreover, if the switch is operated and DNA becomes recognized in its double-stranded form, the unwound S/MAR structure becomes unconstrained and thereby capable of relaxing the positive supercoils. Removal of the former barrier would permit other polymerases to initiate transcription in the wake of the first molecule (Dunaway & Ostrander, 1993). It is noted that this concept is formally similar to the mechanism by which positive supercoils are thought to open chromatin structures (Lee & Garrard, 1991), and it is also related to the scenario described by Dijkwel and Hamlin (1995) in which a S/MAR can only serve the function of an origin of replication if it has experienced a history of transcription.

Conclusions. A prototype S/MAR element from the domain border of an inducible gene can be placed within a transcription unit to augment transcriptional initiation. A prerequisite for this activity is a certain minimum distance downstream from the promoter. If these requirements are met, the need for extended flanking sequences can be obviated and S/MARs can even be used for an improvement of retroviral vectors. One location that is appropriate for S/MARs in these vectors is the LTR which maintains its functions after this modification. The fact that a given S/MAR can, in principle, serve the dual role of a domain border and an internal regulatory element suggests that it is not necessary to postulate the existence of functionally different S/MAR types. These findings are reconciled in a model considering S/MARs as topology-operated switches which can either form a barrier, provide for a topological separation of independent domains, or confer their topological state to support promoter or enhancer functions (Bode et al., 1996).

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