MOLECULAR AND CELLULAR BIOLOGY, May 1990, p. 2302–2307
0270-7306/90/052302-06$02.00/0

Vol. 10, No. 5

The Chicken Lysozyme 5′ Matrix Attachment Region Increases Transcription from a Heterologous Promoter in Heterologous Cells and Dampens Position Effects on the Expression of Transfected Genes

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Received 31 October 1989/Accepted 25 January 1990

Matrix attachment regions (MARs) are DNA elements that dissect the genome into topologically separated domains by binding to a chromosomal skeleton. This study explored the putative influence of the MAR located 5′ of the chicken lysozyme gene on expression of heterologous genes in heterologous cell systems. Expression of a construct with the chloramphenicol acetyltransferase (CAT) indicator gene controlled by the herpes simplex virus thymidine kinase promoter (TC) and a construct in which the same transcriptional unit is flanked by chicken lysozyme 5′ MARs (MTCM) was assayed after stable transfection into rat fibroblasts. Median CAT activity per copy number in MTCM transfectants was elevated approximately 10-fold relative to that in TC transfectants. Total variation in normalized CAT activity decreased from more than 100-fold among TC transfectants to nearly 6-fold among MTCM transfectants. The steady-state level of transcripts and the relative rate of transcription were increased in MTCM transfectants, as shown by S1 nuclease and run-on transcription assays, respectively. The chicken lysozyme 5′ MAR thus can confer elevated, less position-dependent expression on a heterologous promoter in cells of a different species by increasing the density of transcribing RNA polymerase molecules. MAR-mediated transcriptional enhancement suggests that MARs are important for gene expression and not just for DNA packaging.

The chromatin of interphase nuclei and metaphase chromosomes is organized into topologically closed loop-domains (1, 10, 17, 21). These domains are constrained by a nuclear framework called matrix in interphase nuclei (2) and scaffold in metaphase chromosomes (28). The DNA regions where chromatin loops are attached to the matrix (matrix attachment regions [MARs]; often used synonymously with scaffold attachment regions [14]) have been localized to specific sequences within the flanking regions of several genes of *Drosophila melanogaster* (14, 25–27) and of a few vertebrates, such as the chicken lysozyme (30), human beta interferon (4), and β-globin (15, 19, 30a) genes. Most MARs have been characterized as A+T-rich sequences of variable length with an increased density of sequences homologous to the consensus sequence of the topoisomerase II cleavage site. MARs are not only important for structural reasons but may also function in regulation of gene expression, as MARs have been found upstream and downstream of the genes or gene clusters (4, 14, 19, 25, 30). The MARs of the chicken lysozyme gene are located at the boundaries of the "active" chromatin domain (30, 33). Most MARs are located in close proximity to *cis*-acting regulatory DNA elements identified either genetically or functionally (7, 9, 14, 19, 20, 26, 30). This and similar evidence led to speculations that MARs are the limits of larger functional or regulatory domains (14, 30a). MARs from different origins as well as matrix preparations from species as different as yeasts and mice are interchangeable in in vitro DNA fragment binding assays of nuclear matrices (8, 18, 30), suggesting an evolutionary conservation of the mechanisms of MAR attachment (30a).

It has been recently shown that in a homologous system using MARs of the chicken lysozyme gene linked to the lysozyme promoter to stably transfect chicken cells, the MARs increase gene expression about 10-fold (32). We now wanted to answer the question whether MARs can modulate transcription in different species using heterologous promoters such as the herpes simplex virus thymidine kinase gene (*tk*) promoter. Rat fibroblasts were thus stably transfected either with a construct (TC) containing the herpes simplex virus *tk* promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene (*cat*) or with a construct (MTCM) in which this transcription unit is flanked by chicken lysozyme 5′ MARs. Our results indicated that the chicken lysozyme 5′ MAR can confer elevated, less position-dependent expression on a heterologous promoter in heterologous rat cells.

MATERIALS AND METHODS

Constructs. The structure of plasmid TC (22) is shown in Fig. 1. To construct plasmid MTCM, the 2,764-base-pair (bp) B-1–*Sac*I fragment of the chicken lysozyme 5′ MAR (30), blunt ended at the B-1 site, was ligated into the *Sma*I-*Sac*I-cut plasmid TC (23). This construct was cut with *Xba*I before ligation with the blunt-ended 2,953-bp MAR fragment B-1-X1 (30), attached to *Xba*I linkers (23). Plasmid *tk-neo* contains the Tn*5* neomycin resistance gene (5) controlled by the herpes simplex virus *tk* promoter (sequence coordinates −109 to +51) and the small t intron and polyadenylation signals from simian virus 40.

Cell culture, transfection, DNA isolation. Circular plasmid TC or MTCM (45 μg) was mixed with 3 μg of circular

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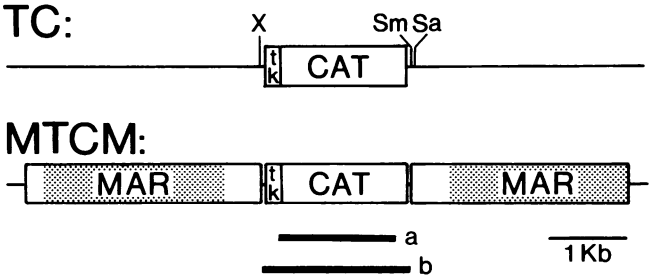

FIG. 1. Constructs used for transfection. Construct TC, previously referred to as pBLCAT2 (22), contains the *cat* gene controlled by the herpes simplex virus *tk* promoter. Construct MTCM is derived from TC and contains, inserted into the *Xba*I (X) site, the chicken lysozyme 5′ 2,953-bp B-1-X1 fragment (28) and, inserted into the *Sma*I-*Sac*I (Sm-Sa) site, the lysozyme 5′ 2,764-bp B-1–*Sac*I fragment. The MARs within both fragments are marked by shaded sections (30). The bar below construct MTCM labeled a shows the extent of the probe (1,525-bp *Hin*dII-*Hin*dII fragment) used for quantitative Southern blotting. The bar labeled b indicates the extent of the *Bam*HI-*Sac*I fragment which was used in the nuclear run-on transcription experiments. Kb, Kilobase.

plasmid *tk-neo* containing the neomycin resistance gene as a selectable gene, and 5×10^5 rat-2 cells (34) were transfected as described previously (12). (In pioneering experiments with solely plasmid *tk-neo*, linearization did not increase the frequency of G418-resistant clones.) The medium was changed after 4 h, and the cells were allowed to recover for 44 h before 800 μg of G418 per ml was added to select for cells that had stably integrated the constructs. G418-resistant clones were isolated 14 days later. Tissue culture conditions used have been previously described (12). Cellular DNA was purified and analyzed (23, 33).

CAT assay. Cells washed twice in phosphate-buffered saline were removed by a rubber policeman in 40 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–150 mM NaCl, pelleted, and stored at −35°C. The pellet was suspended in 0.25 M Tris hydrochloride (pH 7.5), sonified, and cleared by centrifugation. CAT activity was measured by incubating 5 to 80 μl of extract for 15 min to 1 h at 37°C in a total volume of 180 μl containing 0.25 M Tris hydrochloride (pH 7.5), 0.5 mM acetyl coenzyme A, and 0.5 μCi of [^{14}C]chloramphenicol at 60 mCi/mmol (1 Ci = 37 GBq; Amersham Buchler GmbH, Braunschweig, Federal Republic of Germany). After extraction with ethyl acetate, unmodified chloramphenicol and its acetylated derivatives were separated by thin-layer chromatography on silica plates with chloroform-methanol (95:5). After autoradiography or scanning, CAT activity was expressed as picomoles of acetylated chloramphenicol per microgram of protein per hour of incubation time.

S1 nuclease analysis. For S1 nuclease analysis (36), total cellular RNA was prepared by cell lysis in 5 M guanidinium thiocyanate–25 mM sodium citrate–0.5% sarcosyl–0.35% Antifoam A (Sigma)–7% 2-mercaptoethanol (6). RNA was pelleted through a cushion of 5.66 M CsCl–60 mM EDTA (pH 7.0) in a Beckman SW40 rotor at 35,000 rpm and 20°C for 20 h. RNA pellets were dissolved in 1 mM Tris hydrochloride (pH 6.5). For S1 nuclease analysis (36), RNA samples were hybridized to an excess (10 ng) of the 391-bp gel-purified *Eco*RI-*Eco*RI fragment that had been 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase (23) in 400 mM NaCl–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–1 mM EDTA–80% formamide at 50°C for 20 h. After hybridization, probes were diluted with 185 μl of 0.28 M NaCl–0.05 M sodium acetate (pH 4.6)–4.5 mM $ZnSO_4$–21 μg of denatured and sonicated salmon sperm DNA per ml and treated with 800 U of S1 nuclease (Boehringer GmbH, Mannheim, Federal Republic of Germany) for 45 min at 37°C. Reactions were stopped by the addition of 4 μl of 0.5 M EDTA. S1-resistant DNA fragments were extracted with phenol and chloroform and precipitated before analysis by electrophoresis on 4% polyacrylamide–7 M urea sequencing gels (23). Dried gels were exposed to Kodak XAR-5 films.

Run-on transcription experiments. For run-on transcription experiments (37), nuclei were isolated by hypotonic lysis with a Dounce homogenizer (type A pestle) as described previously (24). Nuclei (4×10^6 in 13 μl for each transcription reaction) were suspended at 0°C in 80 μl of transcription buffer (37) (25 mM dithiothreitol, 90 mM KCl, 5 mM $MgCl_2$, 10 mM Tris hydrochloride [pH 7.8], 25% glycerol). A 1-μl portion of a mixture of 25 mM ATP, 12.5 mM GTP, and 12.5 mM CTP and 125 μCi (6 μl) of [α-^{32}P]UTP at 800 Ci/mmol (Amersham) was added to each reaction. The nuclei were incubated at 37°C for 10 min. RNA was isolated by treatment with RNase-free DNase I (69 U) at 37°C for 5 min, followed by proteinase K treatment (11) and extraction with hot phenol-chloroform-isoamyl alcohol as described previously (29). Residual DNA and free nucleotides were removed by a second treatment with DNase I and, after precipitation, by several washes with 70% ethanol–100 mM sodium acetate (11). In a typical experiment, 2.3×10^6 to 3×10^6 cpm of RNA were obtained. For analysis of the in vitro-synthesized RNA, plasmid TC was digested with *Bam*HI and *Sac*I and 2.4-μg aliquots of the resulting fragments were resolved by electrophoresis on a 1.1% agarose gel and then Southern blotted to nitrocellulose (31). Baked filters were hybridized to approximately 3×10^6 cpm of [^{32}P]RNA in a total volume of 0.75 ml as described previously (35), washed, and exposed to Kodak XAR-5 films.

RESULTS

Increased copy number of chicken MAR vector in stable rat fibroblast transfectants. MARs could possibly be of importance during the processing, the integration, or the maintenance of transfected DNA. This was tested with two vectors: one with the *cat* gene linked to the herpes simplex virus *tk* promoter (TC) and the other with two chicken lysozyme 5′ MAR fragments (2,953 and 2,764 bases in length) flanking the *tk-cat* fusion gene in opposite polarities (MTCM) (Fig. 1). In each of the fragments approximately 2.3 kilobases (marked by shaded sections in Fig. 1) have been identified as MAR in previous fine mapping experiments (see Fig. 3 in reference 30). Vectors were transfected into rat-2 fibroblasts together with a plasmid which contained the *tk* promoter linked to the neomycin resistance gene (*tk-neo*) to permit selection of transfected cells in medium containing G418. G418-resistant rat-2 cell clones were isolated 14 days later. A total of 63% of the clones transfected with the construct TC and 52% of the clones transfected with construct MTCM showed correct genomic integration and are listed in Table 1. Copy number of the integrates as determined by quantitative Southern blotting of genomic DNA cut with *Hin*dII and hybridization with a 1,525-bp probe containing the *cat* gene (Fig. 1) revealed that the number of integrated copies varied from 1 to 270. The mean copy number was more than threefold elevated in the clones transfected with construct MTCM relative to those transfected with construct TC, a difference that was found to be statistically significant ($P < 0.05$). After digestion of genomic DNA with *Sal*I, *Kpn*I, or *Pvu*I, which

TABLE 1. Copy number and CAT activity in rat-2 cell clones stably transfected with constructs TC and MTCM

Construct	Clone	Copy no.	CAT activity (pmol/μg of protein per h)	CAT activity/copy (pmol/μg of protein per h)
TC	3/2	1	0.71	0.71
	3/4	1	12.2	12.2
	3/6	1	1.88	1.88
	4/2	1	2.19	2.19
	4/10	4	24.7	6.17
	4/1	6	1.71	0.28
	4/6	6	3.37	0.56
	4/3	8	175.0	21.9
	3/7	36	21.8	0.60
	3/9	54	69.5	1.28
	4/5	54	10.1	0.18
	3/8	72	361.5	5.02
Mean ± SD		20 ± 26		4.41 ± 6.24
Median				1.68
MTCM	6/10	1	10.6	10.6
	6/22	2	26.6	13.3
	6/17	12	325.3	27.1
	5/6	24	400.3	16.7
	6/3	25	236.5	9.50
	6/15	30	1,609	53.6
	6/21	42	1,047	24.9
	6/9	48	548.0	11.4
	6/6	70	645.5	9.22
	6/23	84	2,138	25.4
	6/14	108	3,530	32.7
	5/5	110	1,455	13.2
	6/1	138	1,760	12.8
	5/3	270	4,875	18.1
Mean ± SD		69 ± 72		19.9 ± 11.8
Median				17.2

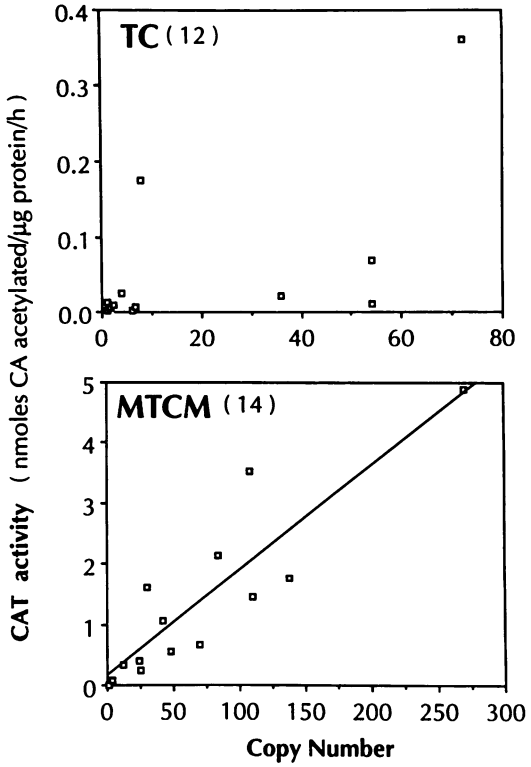

FIG. 2. Analysis of stable transfectants: copy number of *cat* and MAR-*cat* vectors and total CAT activity. Total CAT activities as listed in Table 1 are plotted against the number of integrated plasmid copies as listed in Table 1. CA, Chloramphenicol.

cut once or twice in the constructs but rarely in mammalian DNA, Southern blotting, and hybridization with total construct DNA as a probe, the predominant appearance of the diagnostic full-length (*Sal*I or *Kpn*I) fragments or *Pvu*I-*Pvu*I fragments showed that the majority of the multicopy integrations occurred in tandem in a head-to-tail fashion (data not shown).

CAT activity in stable transfectants with the MAR vector is increased compared with those with the control vector. We determined the CAT activity in the two types of stable transfectants to evaluate a potential influence of the heterologous MARs on expression of the *cat* gene. Table 1 shows total CAT activities in cell extracts as well as CAT activities normalized per integrated plasmid copy. Insertion of the MARs led to a 4.5-fold increase in the mean level and a 10-fold increase in the median level of normalized CAT activity. Statistical analysis by a parametric analysis (Student's *t* test) and a nonparametric test (Mann-Whitney U test) revealed that the increase in expression level is significant ($P < 0.05$). Total variation in normalized CAT activity decreased from more than 100-fold among TC transfectants to nearly 6-fold among MTCM transfectants. A plot of total CAT activities versus copy numbers of integrated constructs (Fig. 2) also indicates the decrease of quantitative variation in *cat* gene expression caused by the presence of flanking MARs. Further, CAT activity in MTCM-transfected clones appeared to be linearly correlated with copy number. These results thus show a positive influence of the chicken lysozyme MAR on expression of heterologous genes (*tk-cat*) in a heterologous species. They further indicate that the sensitivity of *cat* gene expression to the chromosomal integration site is decreased in MAR transfectants.

Steady-state levels of *tk-cat* gene transcripts are elevated in MAR transfectants. To verify that RNA expression of the *tk-cat* gene in the clones transfected with construct MTCM is higher than in those transfected with construct TC, we performed a quantitative S1 nuclease analysis of transcription in three TC- and five MTCM-transfected clones (36). The selected clones (TC, 3/9, 4/5, and 3/8; MTCM, 6/22, 6/15, 6/23, 6/1, and 5/3) showed normalized CAT activities fairly well distributed around the respective median levels (Table 1) and thus constituted a representative sampling of the clones. In addition, the five selected MTCM clones varying in copy number from 2 to 270 represented nearly the whole spectrum of integration frequency. The clones containing MTCM gave high levels of accurate transcription initiation (Fig. 3). In contrast, transcription from the MAR-deficient construct resulted in an activity below the level detectable by S1 nuclease analysis. Using a quantitative analysis by densitometric scanning of the autoradiogram and correction for variations in the amount of RNA utilized, we determined the level of transcripts to be highest in clone 5/3, approximately one-third less in clones 6/15, 6/23, and 6/1, and approximately 1/10th in clone 6/22 (see the legend to Fig. 3). Comparison of these results with those on CAT activity (Table 1) showed that the level of transcripts is proportional to the CAT activity. Minor quantitative differences may be a

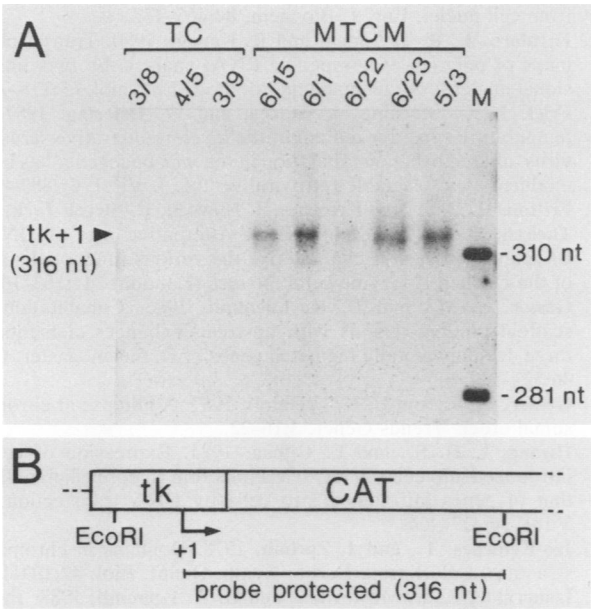

FIG. 3. Quantitative S1 nuclease analysis of *tk-cat* gene transcription in TC- and MTCM-transfected cell clones shows a severalfold increase in the steady-state level of accurately initiated *tk-cat* transcripts in MAR-*tk-cat* compared with *tk-cat* transfectants. (A) Total cellular RNA was prepared from the indicated clones, and 4.9 to 10 μg was hybridized with the 5'-end-labeled 391-nucleotide (nt) *Eco*RI-*Eco*RI probe spanning the *tk* promoter (see panel B). After S1 nuclease digestion, the protected probe was analyzed on a 4% sequencing gel. The start site of the *tk* promoter (tk+1) is indicated. The last lane (M) contains ϕX174 replicative-form *Hae*III digestion marker fragments. Densitometric scanning of the autoradiogram and normalization on equal amounts of input RNA indicated the following levels of relative transcription (defining the hybridization signal obtained with clone 5/3 as 100%): 3/8, 4/5, and 3/9, <3%; 6/15, 77.3%; 6/1, 57.8%; 6/22, 13.7%; 6/23, 61.2%; 5/3, 100%. (B) Diagram of the promoter region of the *tk-cat* constructs. The left box shows the *tk* promoter region from −105 to +51, and the right box shows the upstream portion of the *cat* gene. The arrow denotes the start site of transcription. The probe used was a 391-nucleotide fragment spanning the two indicated *Eco*RI sites.

consequence of the two different methods of measuring expression.

MAR elements increase rate of transcription of linked genes. S1 nuclease analysis detects the steady-state level of stable transcripts. The relative density of nascent RNA transcripts along the *cat* gene was thus determined in a run-on transcription experiment (37) to decide whether MARs increase the rate of *cat* gene transcription. Nuclei were isolated from clones (3/9, 3/8, 6/21, and 6/14) that contained comparable numbers of inserted plasmid copies (54, 72, 42, and 108, respectively) and were incubated for 10 min with [α-^{32}P]UTP to allow endogenous RNA polymerase to elongate nascent RNA transcripts. The purified [^{32}P]RNA was hybridized to a large excess of the Southern-blotted *Bam*HI-*Sac*I fragment containing the *tk* promoter and the *cat* gene (Fig. 4). The hybridization signals obtained with the MTCM-transfected clones 6/21 and 6/14 were approximately three- and eightfold higher, respectively, than those obtained with the TC-transfected clones 3/9 and 3/8, demonstrating that the chicken lysozyme MAR increased the rate of transcription (see the legend to Fig. 4). This result is in agreement with the CAT assays in Table 1 and the S1 nuclease analysis in Fig. 3. It further shows that the chicken lysozyme MAR increased gene expression by an elevation of

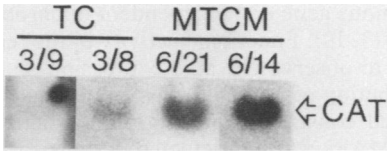

FIG. 4. Run-on transcription experiment shows enhancement of the rate of transcription by the chicken lysozyme MAR. Nuclei were isolated from the indicated clones and incubated in an in vitro transcription system with [α-^{32}P]UTP. Purified [^{32}P]RNA was hybridized to the Southern-blotted *Bam*HI-*Sac*I fragment from construct TC containing the *tk* promoter and the *cat* gene (Fig. 1). Densitometric scanning of the autoradiograms and normalization on equal amounts of input [^{32}P]RNA showed the following relative levels of nascent transcripts (defining the hybridization signal obtained with clone 6/14 as 100%): 3/9, 10.6%; 3/8, 12.8%; 6/21, 33.0%; 6/14, 100%.

the density of transcribing RNA polymerase molecules along the *cat* gene.

DISCUSSION

The results presented here show that matrix attachment regions have a profound influence not only on the structure and association of chromatin with the nuclear matrix, as shown by others (4, 14, 15, 19, 25–27; Phi-Van and Strätling, in press), but also on the expression of a nearby gene in heterologous cells. The 5' MAR of the chicken lysozyme gene when placed 5' and 3' of a *tk* promoter-*cat* construct (i) led to an increase in the expression of the *cat* gene and (ii) dampened the sensitivity of the expression of the transfected *tk-cat* construct to the chromosomal integration site, a feature normally observed for retrovirally transduced genes but not for transfected DNA (16). The increased expression was found at three levels: (i) in nuclear run-on experiments in the synthesis of nascent CAT transcripts, (ii) by S1 nuclease analysis at the steady-state level of CAT transcripts which are properly initiated with the *tk* promoter, and finally, (iii) by measuring CAT activity at the protein level. Generally, total CAT activity, the steady-state level of *cat* transcripts, and the level of nascent *cat* transcripts were proportional to each other in most of the clones analyzed (Table 1; Fig. 3 and 4). One minor exception should not be left unmentioned. Total CAT activities of TC clones 3/9 and 3/8 were determined as 69.6 and 361.5 pmol/μg of protein per h, respectively, and that of MTCM clone 6/22 as 26.6 pmol/μg of protein per h (Table 1). The two TC clones, however, did not yield a hybridization signal in the S1 nuclease analysis, while that of the MTCM clone was weak but clearly detectable (Fig. 3). Reduced stability of CAT in clone 6/22 may be one of the possible reasons for this deviation. In each of the MAR fragments inserted in construct MTCM, 2.3 kilobases (approximately 80%) (marked by shaded sections in Fig. 1) have been identified as MAR in previous fine mapping experiments (30). There is no indication that the remaining non-MAR sequences have a regulatory function (13; unpublished data).

The approximate 10-fold increase in the median level of expression in MAR-*tk-cat*-transfected cells relative to cells which contained the control vector compares favorably with the MAR-induced increase in expression shown recently for the homologous system (32). A β-globin minilocus containing domain-flanking sequences with MARs and regulatory (= nuclease-hypersensitive) sites has been created that exhibits expression which is comparable to the expression of

the endogenous gene and independent of chromosomal integration site (3, 19). Functional activity of the regulatory sites is required to observe elevated expression, but clarification of a contribution of MARs to this effect needs studies with more refined constructs. Transcriptional activation of promoters by MARs was not detected in transiently transfected cells, as shown by the previous work (32), suggesting that MARs (i) exert their influence differently from that of conventional enhancers and (ii) can only react within the normal framework of chromatin, which most likely is a feature of stable transfectants. The major conclusion of this study, however, is that MARs can exert a pronounced effect on gene transcription in cells of different species (rat versus chicken), in cells in different states of specialization (macrophage precursors versus fibroblasts), and in cells with heterologous promoters (lysozyme versus *tk* promoter). This effect of MARs is reminiscent of earlier results by other groups cited previously (8, 18, 30) showing that MARs are also interchangeable in an in vitro DNA fragment matrix binding assay. These studies suggest an evolutionary conservation of the mechanisms of MAR attachment (30a). Our present results, moreover, indicate that the chicken lysozyme 5′ MAR or regions close to the MAR also exert enhancing effects on transcription across the species barrier. The evolutionary conservation of these features indicates that both matrix attachment and transcriptional activation are essential features of MARs.

ACKNOWLEDGMENTS

We thank M. Hallupp and U. Bergholz for skillful technical assistance and G. Schütz and M. Grez for generous gifts of plasmid DNA. We thank B. Nippa for expert typing of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (Str 145/11, Os 31/12).

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