Distribution of transcription factor binding sites along 5'-upstream sequences of genes: estimation of minimal promoters and upstream regulatory regions

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Summary

Precise analysis of gene transcription regulation requires accurate estimation of gene regulatory elements. Transcription factors (TF) binding sites are distributed unevenly along gene 5'-upstream sequences. The analysis of gene 5'-upstream sequences showed the presence of high-density regions of transcription factor binding sites (HDR). The first HDR adjacent to the transcription start point corresponds to a minimal promoter, and the 5'-end of I-HDR corresponds to the 5'-end of minimal promoter. The HDRs located more distant to gene transcription start point often correspond to enhancers and other gene regulatory elements. The common for milk protein genes TF binding sites are located in I- and II-HDR suggesting that tissue- and time-specificity of this group of genes are determined by minimal promoter, enhancer or by both. The analysis of distribution of TF binding sites along gene 5'-upstream sequences suggested that the expression of rabbit α casein gene can be regulated by minimal promoter only. The expression of all analyzed WAP genes; bovine and goat κ casein, bovine and goat BGL, and rabbit α lactalbumin genes are shown to be regulated by an enhancer. The expression of all β casein genes; bovine and rat α ca-

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sein; goat, mice and rat α lactalbumin genes are found to be regulated by both regulatory elements.

Key words:
promoter, enhancer, transcription factor, regulation of transcription, milk protein genes.

1. Introduction

Gene expression involves multiple levels of control, and regulation of transcription is often the most important step. Transcription is usually regulated by the interaction of proteins with cognate DNA binding sites. These DNA elements are organized into regulatory units known as promoters, enhancers or silencers, scaffold attachment regions (SARs) and locus control regions (LCRs). Identification of these elements might assist in the functional classification of genes, predict tissue and time and level of gene expression.

Recently, great efforts have been made to unravel complete genomes. However, draft sequence has poor informational value if it is not accompanied by functional data. One of the most important regulatory elements are promoters. Recent method of estimation of the 5'-end of promoters by sequential deletions of a gene 5'-upstream sequence is laborious and time-consuming. Progress in bioinformatics allows analysis of draft DNA sequences *in silico*. Computer analysis of 5'-upstream sequences needs databases of transcription factor (TF) binding sites and software for searching the sequences for presence of putative TF binding sites in them. There are two databases of TF binding sites: Transcription Factor Database (TFD) (1) and TRANSFAC (2) and several programs for searching the sequences for putative TF binding sites: – SignalScan (3), TESS (4), MatInspector (5) and other. The computer analysis of DNA sequences showed that the density of putative TF binding sites in promoters is higher than in non-promoter sequences (6). The density of transcription factors along 5' upstream sequences is distributed unequally (7). A statistical model, based on clustering of transcription factor binding sites, was successfully applied for the location of regulatory regions in genomic DNA (8). These features of promoters suggests that the density of transcription factors binding sites could be used for the estimation of minimal promoter's boundaries in gene 5'-upstream sequences. As minimal promoter we understand the part of gene 5'-upstream sequence which confers correct tissue and time specific gene expression.

Expression pattern of milk protein genes share several common features: they are expressed only in the mammary gland during late pregnancy, lactation and early involution and their expression are stimulated by synergistic action of three lactogenic hormones: insulin, prolactin and glucocorticoids and are attenuated by progesterone (9). Common expression features suggest the existence of common motifs and a set of transcription factor binding sites. Previously performed computer analysis of entire milk protein gene 5'-upstream sequences revealed several common motifs and TF binding sites (C/EBP, CTF/NF1, MAF, STAT5), however, the loca-

tion of these motifs and binding sites changes from one promoter to another (10). The aim of the present research is the analysis of density of TF binding sites along 5'-upstream gene sequences and their correspondence to known regulatory units. The function of TF binding site density was applied to estimate 5-end boundaries of a minimal promoter.

2. Materials and methods

The analysis of TF binding sites distribution along genes 5' upstream sequences was performed on a training set of 10 genes (tab. 1). The analysis of 5'-upstream sequences consisted of two stages:

Stage I. Stringed-based search of 5'-upstream sequences for the presence of TF binding sites was performed by TESS software against TRANSFAC database. Searching parameters: maximum allowable mismatch: 0%, minimum element length: 6 bp, minimum lg-likehood: 12, secondary Log-likehood density threshold: 1.6. To remove redundancy in output file, the exact binding sequences are referred to that accession number as it appears for the first time in file, name of transcription factor is chosen as more convenient; e.g. below is the present part of output file for bovine αS1 casein gene promoter.

Table 1

Training set of 5'-upstream sequences

Gene	GenBank Acc. No	Length of sequenced 5'-upstream region	Reference
Bovine β casein	X14711	1722	(15)
Rat β casein	M10936	783	(26)
Ovine, β lactoglobulin	X68105	4203	(27)
Rat, prolactin	M10041	2622	(28)
Chicken, fatty acid synthase	X77339	1029	(12)
Human, *c-erb*B2	X56495	3297	(11)
Rat, acyl-CoA-binding protein	X96560	2232	(29)
Bombyx mori, POU-M1/SGF-3	L01266	986	(30)
Mouse, β1,4-galactosyltransferase	L16840	1897	(13)
Human, aldolase A	X12447	4160	(14)

Fragment of original output file:

Inde	M	LLH	LLH Den	Tf AccNo	Sequence	Factor Name
1137	N	18.00	2.0000	R03402	CTACAATGT	DSXF
1994	N	18.00	2.0000	R02238	AGAGGAACT	Pu.1
1994	N	18.00	2.0000	R02238	AGAGGAACT	PU.1
1994	N	18.00	2.0000	R02238	AGAGGAACT	PU.1
230	N	18.00	2.0000	R02168	GGGGGAGGG	H4TF-1
1997	N	18.00	1.2000	R02181	NGAANNGAANNGAAN	HSF1 (short)
1997	N	18.00	1.2000	R02181	NGAANNGAANNGAAN	HSF1 (long)
1997	N	18.00	1.2000	R02181	NGAANNGAANNGAAN	HSF1
1997	N	18.00	1.2000	R02181	NGAANNGAANNGAAN	HSF
1997	N	18.00	1.2000	R02181	NGAANNGAANNGAAN	HSF
1997	N	18.00	1.2000	R02181	NGAANNGAANNGAAN	HSF
379	N	16.00	2.0000	R04032	TGACATCA	c-Jun
379	N	16.00	2.0000	R04032	TGACATCA	c-Fos
379	N	16.00	2.0000	R03479	TGACATCA	AP-1
392	N	16.00	2.0000	R03087	CTGATAAG	GATA-1

R03402 is referred to as DSXM and DSXF, for further analysis the name DSXM was chosen, R02181 is referred to as HSF1 (long), HSF1 (short), HSF1 and HSF was chosen HSF,

Sequence TGACATCA is referred to as the binding sites for TF c-Jun (R04032), c-fos (R03738) and AP-1 (R03479), AP-1 was chosen for further analysis.

Fragment of reformed output file:

Inde	M	LLH	LLH Den	Tf AccNo	Sequence	Factor Name
1137	N	18.00	2.0000	R03402	CTACAATGT	DSXM
1994	N	18.00	2.0000	R02238	AGAGGAACT	PU.1
230	N	18.00	2.0000	R02168	GGGGGAGGG	H4TF-1
1997	N	18.00	1.2000	R02181	NGAANNGAANNGAAN	HSF
379	N	16.00	2.0000	R04032	TGACATCA	AP-1
392	N	16.00	2.0000	R03087	CTGATAAG	GATA-1

Stage II. Computer program WSignal (the source code on C language) was elaborated to search in input sequences the regions with non-random distribution of TF sites, obtained at the first stage.

Model

Let us consider the 5'-upstream sequence, L – length of the region, n_L – the number of TF sites detected.

If TF sites are distributed along 5'-upstream sequence randomly, the probability that any TF site could be assessed is $p_{TF} = n_l/(L-l+1)$, where l – length of TF site. The probability of event: k' sites occurred in the region of length "L", if the sites appear independently, the probability has binomial distribution – there is L'-l+1 possibility for k' appearance:

$$P(k'|L') = C^{L'}_{k'} p_{TF}^{k'} (1-p_{TF})^{(L'-k')} \quad (1)$$

Algorithm for detection of regions:

1. Input sequence is spliced out on the regions: the optimal length of region is estimated after several iterations. "Optimal" length should maximize the number of TF's sites in regions. The probability of TF site occurring in each region is calculated as follows: if k_{wi} – the number of TFs sites in region number i, and L'_{wi} – length of region, the probability of TF site in w_i was $p_{wi} = n_{wi}/l_{wi}$.

2. The left or right frame of region w_i is consequently extended and, simultaneously, the probability p_{wi} recalculated; each frame shift on 1 bp issued recalculation; if the former probability was more than the newly obtained one, the left/right border of the frame fixed and the next region is chosen; else the next bp is taken.

3. After all the regions had been analyzed for each region, binomial probability of simultaneous appearance on the length L' k' TF sites was calculated (1). If thus obtained probability was less or equal 5%, then the region was considered as "non-random" and presumably containing some 'functional' signal.

3. Results

3.1. Localization of nonrandom windows of high-density transcription factor binding sites in training set of 5'-upstream sequences

The analysis of the localization of TF binding sites along 5' upstream sequences displayed their unequal distribution. The localization of the regions with nonrandom high-density TF binding sites in training set of 5' upstream sequences (tab. 1) are present at figure 1. For 10 analyzed sequences, 31 HDR with low probability of random occurrence (<5%) was found. The first region is 200-640 bp length and begins at the distance 0-44 bp from the start point of transcription (with the exception of rat acyl-CoA-binding protein gene, where it begins at -200 bp). This region was almost as long or longer then the experimentally estimated minimal promoter in all analyzed genes. A part of HDR extending more distal than the experimentally estimated minimal promoter also contains regulatory elements important for gene expression. A distal part of *c-erb*B2 HDR has binding sites for TF present

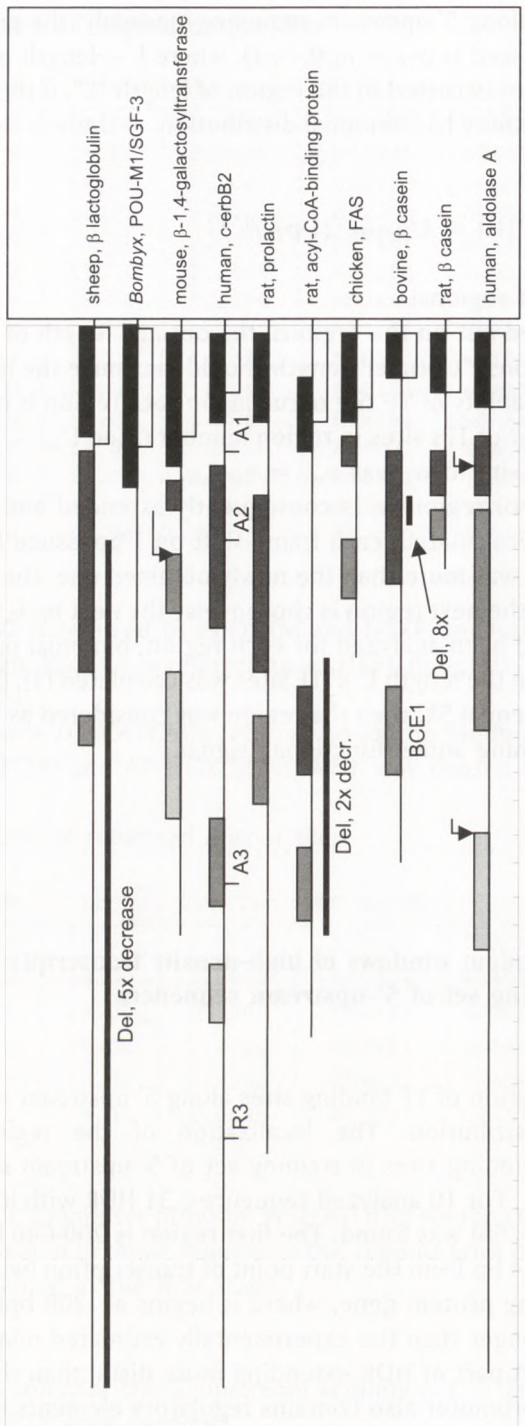

Fig. 1. Localization of high-density transcription factor binding sites in training set of 5'-upstream sequences.

only in some types of cells (11), fatty acid synthase – negative regulatory elements (12), β1,4-galactosyltransferase (13) and aldolase A (14) has alternative promoters. The obtained result suggest that 5' end of I high-density transcription site binding region can be 5'-end of a minimal promoter.

Besides minimal promoter in 5'-upstream sequences, 2-5 distal HDR were found. Most of these regions are also important for the regulation of gene expression. The III-HDR (-1395/-1722) of bovine β casein gene corresponds to BCE1 enhancer (15). Different positive and negative regulatory elements were found in the positions corresponding to II-HDR in β casein (15); II, III and IV HDR in c-erbB2 (11) and, II- and (or) III-HDR in β lactoglobulin genes. The deletion of sequences containing part of II and entire III HDR in β lactoglobulin 5'-upstream downregulated the expression of reporter gene about 5 fold and the deletion of II and III regions in acyl-CoA-binding proteins – 2 fold. Some of the HDR correspond to one of the alternative promoters: – β1,4-galactosyltransferase (13), aldolase A (14). For 15 out of 21 HDR there experimental data indicating their participation in regulation of gene expression were found. The summary results showing relationship between non-random high-density regions in training set of 5' upstream sequences and experimental data are presented in figure 1.

3.2. Localization of nonrandom windows of high-density transcription factor binding sites in milk protein gene 5'-upstream sequences

The set of 24 sequenced milk protein gene 5'-upstream sequences (tab. 2) was analyzed for the localization of putative transcription factor binding sites. The results of this analysis are shown in figure 2. It was found that the density of putative transcription factor binding sites is distributed unequally along 5'-upstream sequences. The I-HDR begins immediately (in 16 out of 24) or at 40- 120 bp from the start point of initiation of the transcription (except mouse β-casein gene, where statistically significant I-HDR begins at 240 bp; statistically non-significant – at 0 – 84 bp). The length of I-HDR varied from very short – 40 – 60 bp (goat and human α lactalbumin respectively) up to 802 bp long. The short I-HDR in goat and human α lactalbumins may be reason the fact that of TF binding sites of which which are located between I- and II-HDR have not been describes so far. The 5'-end of II-HDR is at -200 bp in goat and in -360 in human α lactalbumine respectively. For further analysis was taken I-HDR of goat α lactalbumin 0/-200 and 0/-640 mouse β casein.

Table 2

Milk protein gene 5'-upstream sequences

Gene	GenBank Acc No	Length of sequenced 5'-upstream region
Bovine, αS1 casein	X59856	2121
Bovine, αS2 casein	M94327	2486
Rabbit, α casein	M77195	1341
Rat, α casein	X03584	682
Bovine β casein	X14711	1722
Goat, β casein	M90559	1740
Mouse, β casein	X13484	4835
Rabbit, β casein	X15735	2096
Rat β casein	M10936	823
Bovine, κ casein	M75887	2143
Goat, κ casein	Z33882	2245
Sheep, κ casein	L31372	1219
Goat α lactalbumin	M63868	770
Guinea pig α lactalbumin	Y00726	1173
Human, α lactalbumin	X05153	735
Mouse, α lactalbumin	M87863	546
Rat, α lactalbumin	X00461	1246
Bovine, β lactoglobulin	X14710	2171
Goat, β lactoglobulin	Z33881	2148
Macropus eugenii, β lactoglobulin	L14954	678
Sheep β lactoglobulin	X68105	4203
Mouse, WAP	X79437	4070
Rabbit, WAP	X52564	1798
Rat, WAP	X01153	1199

The I-HDR and II-HDR are separated by low density of transcription factor binding site region. The length of this region varied from 56 bp to 397 bp. These regions are long in all β casein genes and in some other gene 5'-upstream regions: rabbit α casein, mouse WAP and guinea pig α lactalbumin (233 bp-397 bp). For the rest of the investigated gene 5'-upstream sequences, their lengths vary in the 56-180 bp intervals. The lengths of II-HDR are similar to the length of I-HDR and vary from 24 to 800 bp.

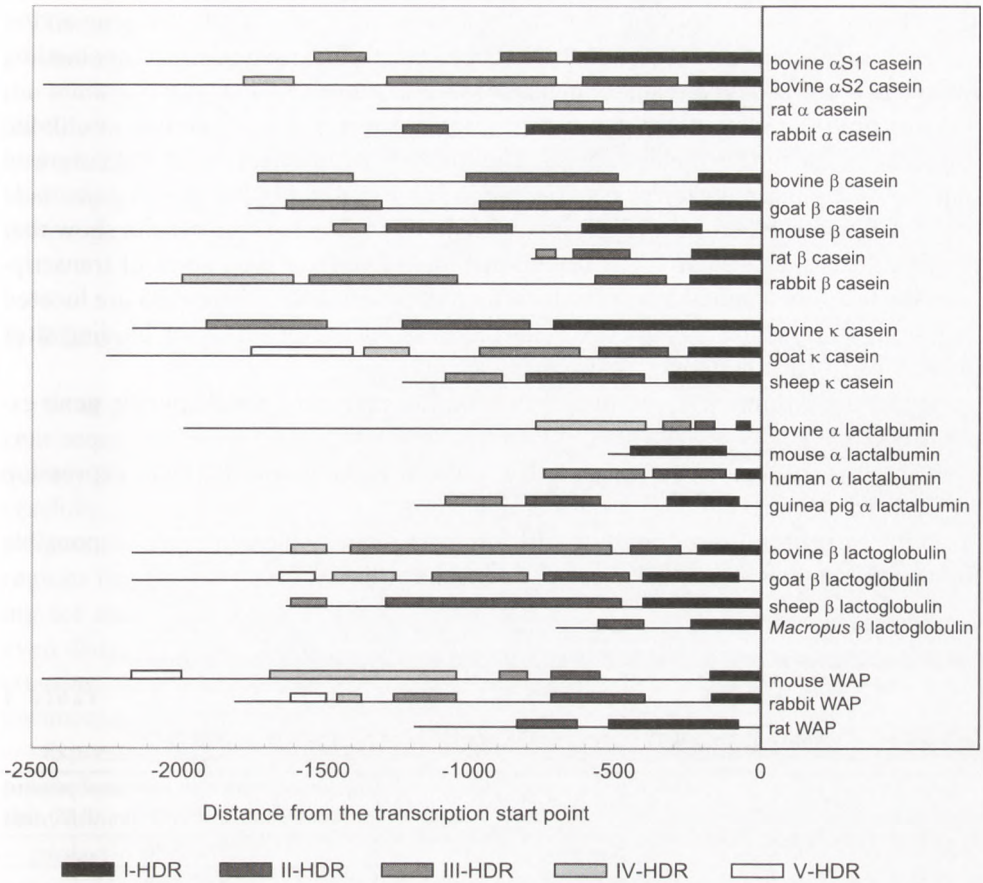

Fig. 2. Localization of high-density transcription factor binding sites in milk protein gene 5'-upstream sequences.

3.3. Localization of putative transcription factor binding sites in milk protein gene 5'-upstream sequences

Previous analysis of milk protein 5'-upstream sequences showed that some transcription factor putative binding sites – C/EBP, CTF/NF, MAF and STAT are common for all of the investigated sequences (10). In this study, we searched milk protein gene 5'-upstream sequences for the presence of putative TF binding sites against the set of TF binding sites collected in TRANSFAC database. There were found binding sites for 146 transcription factors. Additional to previously mentioned TF only c-Ets-2 and half sites of canonical GR binding sites were found in all analyzed 5'-upstream sequences. These TF can be responsive to common regulatory features of all milk protein genes. Binding sites of 45 TF were found only in one of the analyzed 5'-upstream sequences.

This TF may by responsible for specific regulatory features of particular gene and/or not essential for milk protein gene expression. Most of transcription factors binding sites were found in 5'-upstream sequences of several genes.

Only one, the most proximal to transcription start point TF binding site (P-BS) was chosen for further analysis P-BS. The analysis of localization of P-BS showed that the TF common for milk protein genes are located in I-HDRs as well as outside of them. The results of the analysis are presented in table 3. These results show that the first P-BS is located at 17 – 169 bp distances from the start point of transcription, the last one – at 502 – 2117 bp. The length sequence where P-BS are located vary from 265 bp to 2137 bp. The localization of transcription factor binding sites outside of I –HDR supposes that:

a) minimal promoters are not responsible for mammary gland specific gene expression,

b) regulatory regions responsible for mammary gland specific gene expression are located outside minimal promoters,

c) minimal promoters together with other regulatory elements are responsible for mammary gland gene expression during lactation.

Table 3

Localization of putative 5' regulatory region in milk protein gene 5'-upstream sequences

Species	Gene	Localization of the start point most proximal to transcription TF binding sites (P-BS) (bp)
Cow	αS1 casein	-127/-1930
	αS1 casein	-159/-1819
	β casein	-169/-1669
	κ casein	-40/-1484
	β lactoglobulin	-126/-1587
Goat	β casein	-117/-1653
	κ casein	-19/-2136
	α lactalbulmin	-172/-714
	β lactoglobulin	-102/-1803
Mouse	β casein	-95/-1367
	α lactalbulmin	-17/-502
	Whey acidic protein	-56/-628
Rabbit	α casein	-22/-691
	β casein	-141/-2082
	α lactalbulmin	-18/1278
	Whey acidic protein	-48/-1376
Rat	α casein	-18/512
	β casein	-90/-355
	α lactalbulmin	-66/-916
	Whey acidic protein	-87/-626

The analysis of distribution of the P-BS along 5'-upstream sequences show that all three possibilities could take place. All P-BSs of common TF are located inside the minimal promoter of rabbit α casein gene. All PB-Ss of common TF are located outside minimal promoters in 9 genes: all WAP; bovine and goat κ casein; bovine and goat BGL, and rabbit α lactalbumin. Common TF binding sites are located in both minimal promoter and outside of them in bovine and rat α casein, all β casein genes, goat, mice and rat α lactalbumin genes.

4. Discussion

Gene promoters have high density of TF binding sites in comparison with adjacent sequences (6). A simple statistical model of limited specificity based on the frequency profile of TF-sites in promoter and non-promoter sequences has been successfully used in a software to locate polymerase II promoters (16). More advanced program FunSiteP uses the uneven distribution of TF binding sites in promoter subregions for promoter recognition (17). Our results, obtained in the analysis of training set and milk protein gene 5'-upstream sequences, also show elevated and uneven distribution of TF binding sites in gene 5'-upstream sequences. Moreover, decreasing of TF binding sites on distal site of I-HDR satisfactory correlates with experimentally estimated 5'-end of minimal promoter.

Expression patterns of milk protein genes share common features: they are expressed only in the mammary gland during late pregnancy, lactation and early involution, their expression is stimulated by synergetic action of three lactogenic hormones – prolactin, glucocorticoids, insulin and attenuated by progesterone (9). Common expression features suggest existence of common motifs and/(or) set of transcription factor binding sites. Similarity of different milk protein gene 5'-upstream sequences in the same species estimated by multiple alignment is low and does not depend on proximity of analyzed fragment to start point of transcription (10).

The analysis of distribution of TF binding sites along milk protein gene 5'-upstream sequences showed the presence of several high-density regions. Similar complex structure was described for osteocalcin gene promoter. More then 600 bp long sequence were subdivided for proximal, distal and far distal promoter in this investigation (18). Location of positioned nucleosome between proximal and distal osteocalcin promoter can suggest that similar events could occur in milk protein gene promoters between TF high-density regions, as well.

The analysis of localization of TF binding sites common for all milk protein gene 5'-upstream sequences showed that they could correspond to minimal promoter, distal regulatory element or both of them. Recently, there have been enhancers for bovine β casein (19,20), human β casein (21) and rabbit α casein genes (22) described. It was shown that the interaction between promoter and enhancer is important for rabbit α casein gene expression (22).

Considerable variety of transcription factors binding sites found in milk protein genes 5'-upstream regions suggests that the group of genes can be transcribed with low efficiency not only during pregnancy and lactation and not only in mammary gland epithelial cells. Trace expression of β casein has been reported in the mammary gland of virgin mice cycled in the estrus stage of the estrous cycle (23) and in the virgin Wistar-Kyoto rat (24). Weak expression of WAP was found in many tissues (25). It is necessary to note that these observations do not decrease the importance of milk protein genes as a useful model for studies of transcriptional mechanisms in mammals.

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