A Single Base Pair Deletion from the Inactive Octamer-like Motif of the 7S K Distal Sequence Element Brings Full Functionality in Vivo*

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Octamer sequence elements were analyzed for their capacity to induce the 7S K "core" promoter in vivo. The U6 distal sequence element (DSE) which contains a consensus sequence octamer, was able to support efficient 7S K expression in vivo. In contrast, no such function could be attributed to the octamer-like element alone, which is present within the 7S K DSE. However, conversion of this octamer-like element (ATTTAGCAT) to the octamer consensus sequence ATTTTGCAT generated a potent DSE, even in the absence of the CACC box, which constitutes the major functional element of the 7S K DSE. Both the consensus and the octamer-like sequences revealed no cooperativity with the CACC box. Together, these results demonstrate that the octamer-like element of the wild-type 7S K DSE is definitely not functional in vivo. Furthermore, our experiments indicate that in contrast to the RNA polymerase II-transcribed small nuclear RNA genes, in intact cells a single functional DSE motif is necessary and sufficient for maximal transcription by RNA polymerase III of the 7S K RNA gene.

The recent availability of several eucaryotic genes (in addition to 5 S and tRNA) transcribed by RNA polymerase III led to exciting insights into the mechanism of transcription regulation of class III genes (7-13). These genes are transcribed by RNA polymerase II (7-10), EBER RNA (11), 7S K RNA (17), and 7SL RNA (14) genes. It has shown that the effect of these class III genes DSEs can be observed in vivo only. Deletion mutants lacking the DSE showed full activity in vitro.

The octanucleotide sequence ATGGATAAT (18) has been identified as a cell type-specific transcriptional element of the Ig promoter (19-22) and was also found in a number of genes expressed in a non-cell-specific manner such as histone H2B and snRNA genes (23-31). These genes are transcribed by RNA polymerase II. However, the finding of apparently functional octamer elements upstream of the U6 and 7S K RNA genes (7-10, 12, 13) indicated that the octamer motif also can be involved in RNA polymerase III-catalyzed transcription. Efficient transcription of 7S K RNA appears to be regulated differently in vitro and in vivo. Transcription and footprinting analyses performed with purified octamer transcription factors (OTFs) revealed evidence for an essential role in transcription regulation of several octamer-like sequence elements of the human 7S K RNA gene in vivo (13). In contrast, expression studies in intact HeLa cells revealed no direct evidence for an involvement of the octamer-like sequences located upstream of the 7S K RNA gene, although a covered contribution could not be excluded from those data. Rather, a CACC box was identified as the essential element required for efficient 7S K expression in vivo (17).

Here, we have analyzed whether the consensus sequence octamer of the U6 DSE can activate the 7S K "core" promoter and whether the octamer-like sequence of the 7S K wild-type DSE or a consensus sequence octamer generated within the same nucleotide environment is able to interact synergistically with the major 7S K enhancer element: the CACC box.

MATERIALS AND METHODS

Construction of 7S K Mutants—The basic construct with the full 7S K promoter fused to a pAT153 fragment supplemented with the 7S K termination region (4) and the −111 mutant as well as constructs CACmut, CACpse, and CACmut have been described previously (17). Constructs Octa I, II, and III were obtained by cloning a double-stranded synthetic oligonucleotide fragment between the Psfl and SphI restriction sites of the wild-type promoter, of CACmut, and of CACpse, respectively. Likewise, to obtain constructs wt +5 and Octa +5, synthetic oligonucleotide fragments were inserted between the Psfl and BamI sites of the wild-type promoter or Octa I. All constructs were verified by sequence analysis using the dyeoxy chain termina- tion method (32).

In Vitro Transcription—S-100 extracts were prepared from HeLa

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cells as described by Well et al. (33). \textit{In vitro} transcription reactions were carried out with 1.5 \mu\text{g} of supercoiled template DNA and 20 \mu\text{l} of extract in the presence of 0.5 \mu\text{g/mL} of \alpha-amanitin, in a total volume of 50 \mu\text{L} as described previously (4). Reactions were terminated by adding 0.25\% sodium dodecyl sulfate and 250 \mu\text{g} of proteinase K. Phenol extraction and electrophoresis in 6\% polyacrylamide gels in the presence of 8 \text{mM} urea. Exposure of dried gels to Fuji x-ray films was for the times indicated, using Cronex intensifier screens.

\textit{Transfections and S1 Nuclease Analysis of Transcripts—}Transfection of HeLa monolayer cultures and S1 nuclease mapping of RNA synthesized \textit{in vivo} was as described (17). Briefly, purified plasmid DNA was transfected by the calcium phosphate co-precipitation technique (34) using 25 \mu\text{g} of DNA/9-cm plate. For normalization of expression rates, 12.5 \mu\text{g} of a reference plasmid was included in each reaction. This reference plasmid was obtained by deletion of the Asp718\textsuperscript{14} (5'-C11-3') fragment of the wild-type construct and religation of the filled ends. This clone represents the full 7S K promoter and codes for a shortened reference transcript (minus 33 nucleotides) which can be detected by S1 nuclease protection with the same labeled RNA as used for the analysis of construct transcripts. The reference transcripts constantly give rise to a double band (arrowheads) which is caused by an A/T-rich region close to the 5' end of this shortened RNA. S1 nuclease mapping was exactly as outlined previously (4). For quantitation, protected bands were cut from the gel and radioactivity determined in a liquid scintillation counter after background subtraction. All values were normalized for expression of the cotransfected reference plasmid. Relative transcription levels were determined in a liquid scintillation counter after background subtraction. All values were normalized for expression of the co-transfected reference plasmid. Relative transcription levels were determined in a liquid scintillation counter after background subtraction. All values were normalized for expression of the co-transfected reference plasmid.

\textbf{RESULTS}

\textit{In Vitro Transcription of 7S K Mutants That Contain Altered DSEs—}The complete sequence of this human 7S K RNA gene (38) and the essential elements of this gene-external pol III promoter (4) including the distal sequence element (DSE) around position -222 (17) have been described in detail previously. DSE mutant constructs were analyzed for their capacity to direct \textit{in vitro} transcription under the control of the human 7S K core promoter. As is evident from the sequences shown in Fig. 1, these mutations affected the two known sequence elements identified within the DSE; the CACCC box at -222 and the octamer-like sequence at position -232. Although construct CACmut contained a mutated CACCC box (CACCC to gAtCg), in construct CACdel this element was completely removed and replaced by an unrelated vector sequence. O1mut represents a double point mutation of the octamer-like element of the 7S K DSE. In construct wt +5, a 5-bp duplication of the CATG \textquotedblleft spacer\textquotedblright~(-226 to -230; overlapping the 3' half of the octamer-like element and the first bp of the CACCC box) sequence was inserted which separates the two motifs.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig2.png}
\caption{\textit{In vitro} transcription of 7S K DSE mutant constructs. The constructs of Fig. 1 were transcribed in S-100 extracts \textit{in vitro} in the presence of 0.5 \mu\text{g/mL} \alpha-amanitin, and the transcripts were analyzed by S1 nuclease protection of labeled DNA. Protected bands were obtained with the wild-type construct (lane 1) or constructs CACmut (lane 2), CACdel (lane 3), O1mut (lane 4), wt +5 (lane 5), O1 del (lane 6), O1 mut (lane 7), O1 del (lane 8), and the -111 deletion mutant (lane 9), respectively. Lane 10 shows the transcripts of the reference plasmid used for normalization of \textit{in vitro} expression rates (Fig. 3). Exposure of the dried gel to the x-ray film was for 16 h. m, DNA marker fragments.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig1.png}
\caption{Sequences of 7S K DSE mutants. The DSE of the 7S K wild-type gene construct was replaced by synthetic double-stranded oligonucleotide fragments. Mutated sequences are \textit{underlined}, and \textit{lowercase letters} indicate nucleotide exchanges in comparison with the wild-type sequence. Octamer motifs and the CACCC element are marked by \textbf{bold letters.}}
\end{figure}

The DSE of construct Octa I differs from the wild-type sequence only by deletion of a single base pair (-232) which results in a change of the octamer-like element to a consensus sequence octamer binding site. Constructs Octa II and III represent the same CACCC box mutations as in CACmut and CACdel; however, in addition they contained the consensus octamer motif of Octa I. Construct Octa +5 contains the same 5-bp duplication as wt +5, in this case, however, separating the consensus sequence octamer from the CACCC box.

These constructs were analyzed by \textit{in vitro} transcription in cell-free S-100 extracts followed by S1 nuclease protection analysis of specific transcripts. Fig. 2 (lanes 2–8) shows that all these constructs were efficiently transcribed \textit{in vitro}. Normalization to a reference transcript (arrowhead; see \"Materials and Methods\") revealed virtually no differences in \textit{in vitro} transcription efficiency. Furthermore, these efficiencies were comparable to those of the 7S K wild-type promoter (lane 1) or a -111 deletion mutant (lane 9). These results are in agreement with our previous data (17) which revealed that under our conditions a transcriptional activating effect of the 7S K DSE is not observed in cell-free extracts \textit{in vitro}. Lane 10 of Fig. 2 shows \textit{in vitro} transcription of the reference plasmid alone. All these 7S K-specific transcripts were obtained in the presence of low concentrations (0.5 \mu\text{g/mL}) of \alpha-amanitin. In contrast, 150 \mu\text{g/mL} \alpha-amanitin completely inhibited the synthesis of these RNAs (not shown). These results are in full agreement with previous data on RNA polymerase III-catalyzed \textit{in vitro} transcription of 7S K RNA in isolated HeLa cell nuclei (36) or in reconstituted systems (37, 38).

\textit{In Vivo Analysis of 7S K DSE Mutants Containing a Consensus Sequence Octamer—}Since the U6 RNA gene is also transcribed by RNA polymerase III and, in addition, reveals a very similar promoter structure as compared with the 7S K
gene, we cloned a 30-bp sequence centered around the octamer consensus element of the DSE of the human U6 snRNA gene upstream of position -212 of the 7S K promoter. After transfection into HeLa cells, this chimeric class III promoter was analyzed for specific transcripts by S1 nuclease protection of labeled DNA. Since pronounced variations are observed in such transfection experiments, all in vivo analyses were normalized to the transcription rate of a co-transfected reference plasmid, as described under “Materials and Methods.” As is shown in Fig. 3 (lane 2), efficient transcription was obtained under control of this U6/7S K fusion promoter. Normalization to the reference transcripts (arrowheads) revealed that the chimeric promoter was slightly more active (120%) than that of the 7S K wild-type gene (lane 1). The negative minus DSE control (−111 deletion) is hardly detectable (lane 3). These data demonstrate that the U6 DSE is fully active in conjunction with the 7S K core promoter.

On the other hand, our previous study on 7S K transcription regulation in vivo had shown that deletion of the CACCC box virtually eliminated DSE function (17) although such a construct still contained the octamer-like sequence element of the wild-type DSE (see sequence CACael of Fig. 1). This discrepancy concerning the functionality of different octamer elements in the context of the 7S K promoter might be explained by an additional active element being present in the U6 DSE which was transferred to the construct. Therefore, we decided to convert the octamer-like sequence element of the 7S K DSE into a consensus sequence by deletion of the central A-T base pair (from ATTTAGCAT to ATTTGCAT; construct Octa I). Fig. 4 summarizes the results obtained in vivo with constructs containing this consensus sequence octamer, in comparison with those with the octamer-like element of the wild-type DSE. Lane 1 represents the full activity standard of the 7S K wild-type DSE, and lane 2 reflects the result of the consensus sequence octamer within the otherwise unaltered nucleotide environment of the 7S K DSE (Octa I). This comparison showed no effect in relation to the presence of the consensus octamer motif. Very different results emerged if this authentic octamer was analyzed in the absence of a functional CACCC box, in comparison with the octamer-like element of the wild-type DSE. In agreement with the experiments described before (17), mutation of the CACCC box alone (CACmut) resulted in a significant loss of DSE function down to about 20% (Fig. 4, lane 3). However, in this case the presence of the consensus sequence octamer (Octa II, lane 4) restored DSE function almost to full activity (73%). Likewise, the nearly complete inactivation of construct CACael down to 5% basal level activity (lane 5) again was significantly suppressed by the presence of the authentic octamer motif (Octa III, lane 6). Compared with the −111 deletion mutant (lane 7), the DSE function of Octa III was restored to nearly full activity, with an overall expression rate of 69%. These results demonstrate that an isolated consensus sequence octamer constitutes a potent DSE for activation of 7S K RNA

![Fig. 3. In vivo transcription from a chimeric 7S K/U6 promoter](image)

**Fig. 3.** In vivo transcription from a chimeric 7S K/U6 promoter. The DSE of the wild-type 7S K construct was replaced by 30 bp of the U6 DSE, centered upon the octamer motif located at position −220 of the human U6 RNA gene. The DSE of this chimeric promoter now reads 5′-CAGgATTTGcatATCatATcatGTCAT-AcatGGC-3′ with the underlined nucleotides representing U6 sequences. Lowercase letters indicate deviations from the 7S K DSE sequence. The consensus octamer is indicated by bold letters. This chimeric promoter was analyzed for expression in vivo (lane 2) in comparison with the WT construct (lane 1) and the (minus-DSE) −111 upstream deletion (lane 3). Normalization for the reference transcripts (arrowheads) revealed that in this context the U6 DSE was slightly more active (120%) than the 7S K DSE (100%). The −111 deletion again was hardly detectable, and in this particular experiments showed 5.4% activity. Exposure of the gel was for 3 days using an intensifier screen.

![Fig. 4. Functional analysis in vivo of a consensus sequence octamer in the presence or absence of other 7S K DSE elements.](image)

**Fig. 4.** Functional analysis in vivo of a consensus sequence octamer in the presence or absence of other 7S K DSE elements. A, the effect on in vivo expression of a 7S K DSE containing a consensus sequence octamer (Octa I, lane 2) was analyzed in comparison with the octamer-like element of the wild-type construct (lane 1). Lanes 3 and 4 (CACmut and Octa II, respectively) show the analysis of the two octamer sequences in the presence of the mutated CACCC box whereas in lanes 5 and 6 (CACael and Octa III, respectively) the CACCC box was completely deleted. Again, the −111 deletion mutant was included as a basal level control (lane 7), and the arrowheads mark the bands representing transcripts from the cotransfected reference construct. Exposure of the dried gel to a Fuji x-ray film was for 2 days. B, the radioactivity associated with the protected bands of panel A was normalized for the reference transcripts and is presented schematically as percentage of the wild-type control. m, DNA marker fragments.
transcription in vivo. In contrast, no such activating function can be attributed to the octamer-like sequence of the 7S K wild-type DSE alone, whether the CACCC box is present or not.

However, a minor synergistic contribution of the octamer-like element cannot be excluded from this analysis. Therefore, we separated these octamer elements from the CACCC box by a 5-bp duplication, as shown in construct wt +5 and Octa +5 (see Fig. 1). The in vivo analysis of these mutants revealed no decreased transcription activity; instead a slight increase to 115–120% was reproducibly observed with both constructs (Fig. 5), which may reflect an altered DNA structure of the DSE region. These results exclude a cooperativity between the CACCC box and both octamer sequence elements. Furthermore, it appears that the DSE of the 7S K and U6 RNA genes contains a single functional motif and, therefore, is much simpler in structure than those of the U snRNA genes transcribed by RNA polymerase II (30, 31).

Unchanged Specificity for RNA Polymerase III—Switching experiments described for the U6 snRNA gene external promoter resulted in a change in RNA polymerase specificity (see the Introduction). Therefore, we wanted to see if similar effects might occur as a consequence of the presence of a functional octamer sequence. Aside from its intermediate sensitivity toward α-amanitin, the pol III transcription system is also characterized by its specific termination reaction (1, 16). Therefore, to exclude a change in RNA polymerase specificity, experiments were performed with the constructs described before, and 3′ ends of in vitro and in vivo transcripts were determined. Panel A of Fig. 6 shows an S1 nuclease protection analysis of 3′ end-labeled DNA with in vitro transcripts obtained from various constructs. Again, all the constructs analyzed revealed very similar in vitro transcription efficiencies. Furthermore, irrespective of the structure of the DSE present, the same 3′ end of transcripts was observed, which exactly corresponded to the position of the pol III-specific termination sequence of the 7S K RNA gene. Together with the unaltered response toward α-amanitin, this indicates that no switch in RNA polymerase specificity did occur. The maxigene construct (for details see Ref. 4) shown in lanes 3 and 8 of Fig. 6 indicates that termination did not just take place at a fixed distance from the transcription start site, but indeed was dependent on the position of the pol III termination signal. The same holds true for the expression of these constructs in HeLa cells in vivo. No difference in 3′ end formation was observed with constructs containing the consensus sequence octamer (Octa I, II, and III; lanes 9–11), in comparison to the wild-type promoter (lane 7), maxigene (lane 8), and −111 deletion (lane 12; the basal level transcript again is hardly detectable) 7S K constructs. Therefore, changing the DSE structure did not result in a concomitant change in RNA polymerase specificity.

**DISCUSSION**

Our previous results on in vivo expression of 7S K promoter constructs identified a CACCC box as the major upstream activating sequence element of the human 7S K RNA gene (17). On the other hand, the analysis of mutations severely affecting the two octamer-like sequence elements located at −130 and −232, respectively, revealed no hints for a functionality of these elements present within the 7S K upstream region in transcription regulation. Here we have extended our studies on functional sequence elements of the 7S K DSE to the role of the octamer-like sequence element just upstream of the essential CACCC box. This analysis was necessary for
two reasons. First, by analyzing the upstream enhancer of a human U1 snRNA gene, Roebuck et al. (30) described a cooperativity between an essential SPH motif (which alone is capable to stimulate transcription from the U1 core promoter) and an octamer motif located immediately upstream (which by itself lacks this ability). Second, Murphy et al. (13) described transcription activation of the 7S K promoter by purified OTF proteins \textit{in vitro}.

Why are potential octamer factor binding sites dispensable for proper promoter function \textit{in vivo}, whereas transcription from the same promoter \textit{in vitro} can be activated by purified OTFs (13)? The most likely explanation is that the \textit{in vitro} system does not accurately reflect the mode of gene regulation observed in intact cells. In particular, the ratio template \textit{versus} transcription factor may be critical. It is quite conceivable that a highly enriched fraction of purified octamer transcription factor may be able to stimulate transcription \textit{in vitro}, provided potential (though possibly degenerated) OTF binding sites are available. In this case, binding to the octamer element and, consequently, stimulation of transcription \textit{in vitro}, would reflect an unspecified event, resulting from an unphysiologically high concentration of OTFs. Such a loss of specificity \textit{in vitro} was also observed with an Ig promoter, which is poorly transcribed by HeLa cell extracts. In that case, \textit{in vitro} transcription was effectively stimulated by the addition of either the ubiquitous octamer binding factor NF-A1 or the B-cell-specific NF-A2 octamer binding protein (39). The only difference observed upon addition of these two factors, which are highly specific in \textit{in vivo}, was a quantitative one, with the B-cell factor being twice as effective as NF-A1 (39). These experiments point to a considerable loss of specificity of purified octamer factor \textit{in vitro}.

\textit{Consensus Sequence Octamer Versus Octamer-like Element}—Our \textit{in vivo} experiments analyzed the effect of the octamer-like sequence element of the 7S K wild-type DSE, as compared with that of a consensus sequence octamer at the very same position and nucleotide environment of the DSE. The results obtained under our conditions allow three major conclusions.

1. In its wild-type form the octamer-like element is not functional by itself whereas a consensus sequence octamer alone \textit{in vivo} definitely is.

2. Neither the functional consensus sequence octamer nor the octamer-like element of the wild-type DSE seems to be involved in a synergistic interaction with the major sequence element of the DSE: the CACC box element.

3. In comparison with the pol II-transcribed U snRNA DSEs (31), the 7S K DSE apparently is much simpler in structure, with a single functional element being sufficient for transcription activation \textit{in vivo}.

Of course, one might argue that expression of a calcium phosphate-transfected and plasmid-based 7S K gene might represent a fairly artificial system which might not necessarily reflect the "true" \textit{in vivo} situation. We would like to emphasize, however, that comparable results were obtained upon lipofection of plasmid DNA or by using other recipient cells, such as Chinese hamster ovary cells or the hepatoma line HepG2. Therefore, we conclude that our results indeed reflect the mode of regulation controlling the expression of the endogenous 7S K RNA genes.

Our observation that the octamer-like element of the 7S K DSE apparently is nonfunctional is in agreement with previous studies which revealed that a single transversion at any position is sufficient to interfere markedly with both OTF binding and promoter activity of a \(\beta\)-globin construct transcribed by RNA polymerase II (40, 41). Furthermore, the octamer-like sequence element of the 7S K DSE contains 1 additional base pair which exactly separates the two halves of the otherwise native octamer motif. Thus, in comparison with the consensus sequence octamer, this insertion affects the structure of the resulting octamer-like element far more severely than any single point mutation. In addition, as shown in Fig. 4, the octamer-like element by itself was inactive in DSE function, as compared with the consensus sequence octamer, in the same sequence context. This finding also excludes the possibility of a compensating effect of the flanking sequences present within this DSE to restore the functionality of a degenerated octamer, as observed with a number of class II genes (42, 43).

Finally, a recent study (44) on an immunoglobulin V, promoter revealed that a divergent octamer motif may be functional, provided a compensating upstream element is present for interaction. However, our results obtained with the 5-bp duplication (between octamer sequence elements and CACC box) provided no hint for such a synergistic effect and, therefore, would argue against such an interaction. The observation that in the absence of a functional CACC box, the consensus sequence octamer did restore 7S K gene expression only to about 70% may rather reflect a highly artificial DSE structure. Aside from the change in DNA structure (CACC box mutation), the consensus sequence octamer as the only functional element left is not located at the precise position of the original CACC box. Such minor reductions in expression rates are also observed upon relocation of the CACC element. However, assuming that in case of the 7S K DSE a 5-bp insertion did not distort a putative interaction between two adjacent factor binding sites, the only partial rescue by the consensus octamer may point to a hidden interaction of the CACC box with nearby sequences.

The finding that a consensus sequence octamer can activate the 7S K core promoter is in good agreement with the situation observed in the U6 RNA gene. Thus, it appears that the very similar 7S K and U6 core promoters are under control of two different DSEs, both of which very likely are constituted by a single transcription factor binding site. At present, experiments are being performed to characterize in detail the CACC box binding protein which is necessary and sufficient to activate the 7S K core promoter \textit{in vivo}.

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