Volume 17 Number 21 1989 Nucleic Acids Research

Functional analysis of the long terminal repeats of *Drosophila* 1731 retrotransposon: promoter function and steroid regulation

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Received May 24, 1989; Revised September 5, 1989; Accepted September 26, 1989 EMBL accession no. X07656

ABSTRACT

1731 is a *Drosophila* retrotransposon whose transcripts decrease in *Drosophila* cells after treatment by the steroid hormone 20-hydroxyecdysone (20-OH). Several constructions have been made where the bacterial chloramphenicol acetyltransferase (CAT) gene is put under the control of either the 5′ or the 3′ long terminal repeats (LTRs) of 1731. CAT activity assays in transfected Drosophila cells show that either the 5′ or the 3′LTR constitutes a unidirectional promoter. Analysis of partially deleted LTR suggests the presence of so-called silencer and activator regions in these LTRs. Moreover, the first 260 bp of the LTR are sufficient to provoke 20-OH inhibition whereas the first 58 bp are necessary for hormonal responsiveness. These 58 bp contain sequences showing similarities with the targets of trans-acting factors such as Octa1-c and NFkB.

INTRODUCTION

20-hydroxyecdysone (20-OH) is a steroid hormone which has a prominent part in cellular differentiation and development of Insects (1). At a physiological concentration (0.1μM), this hormone induces a wide variety of responses in embryonic *Drosophila melanogaster* cells cultured in vitro. Among them, positive responses such as induction of polypeptides (2,3) or enzymatic activities (e.g. acetylcholinesterase (4,5), β-galactosidase (6), catalase (7), superoxyde dismutase (8)) have been reported (for a review see 9).

Using the transcripts of these steroid responsive cells,we isolated 1731, a retrotransposon with 20-OH negatively regulated expression (10). Retrotransposons are moderately repeated and dispersed genetic transposable elements (transposons) which share many characteristics of retroviruses (see 11−14 for reviews). The main difference with retroviruses seems to be their strictly intracellular localization giving rise to non infectious virus-like particles (VLPs). They have been found in the genome of all eukaryotes examined so far, from yeast to man. They are known to provoke mutations by insertion inside structural genes and to perturb the expression of flanking genes. Such properties have suscitated discussion of a putative role in cancerogenesis. Their function during cellular differentiation and development remain however a subject of controversy.

1731, whose complete nucleotidic sequence has been determined (15), is unidirectional and fully transcribed in a polyadenylated messenger RNA and its transcripts decrease under hormonal treatment. The 1731 long terminal repeats (LTRs) are 336 bp long and present transcriptional signals (TATA-box, polyadenylation signal and possible polyadenylation site). The relative order of these signals is characteristic of proviral LTRs and corresponds to the U3-R-U5 structure (10, 15). Moreover, the LTRs present sets of hexanucleotides (10) similar to those found in the HRE (hormone responsive elements) of steroid hormone

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modulated genes (16,17). This suggests that the promoter activity and the hormone regulatory sequences are located in the LTRs. We thus hypothesize that the 20-OH receptor (18−20) and different transcription factors could bind the LTR. We report here the analysis of recombinant 1731 LTR/chloramphenicol acetyl transferase genes by transient expression in *Drosophila* cultured cells.

MATERIAL AND METHODS

Construction of vectors

Plasmid DNA was extracted by lysis under alkaline conditions as previously described (10). Purified DNA was digested with restriction enzymes under conditions specified by the suppliers. DNA fragments were separated by electrophoresis on agarose gel in 40mM Tris.HCl pH8, 20mM NaAcetate and 2mM EDTA. The desired DNA bands were cut out of the agarose gel and crushed in one volume of Tris 10mM pH8, EDTA 1mM. One volume of phenol was then added. After vortexing, the mixture was placed for 15 minutes in liquid nitrogen. The aqueous phase was recovered by centrifugation and reextracted once with phenol and once with chloroform-isoamylic alcohol (24-1); it was then precipitated with ethanol. These restriction fragments were repaired with T4 DNA polymerase (Amersham) to obtain blunt ends.

The HindIII/ApaI 729 bp restriction fragment of subclone pFP1c (10), which contains the 5'LTR of 1731 preceeded by a 385 bp Drosophila genomic sequence and followed by an 8 bp internal 1731 sequence, was subcloned in both the sense (+) and antisense (−) orientations (subclones B9 and B26 respectively) into the SmaI restriction site of pCAT12 vector polylinker. This vector is derived from pUC12 and pCAT0 (21); it contains the bacterial chloramphenicol acetyl transferase (CAT) coding gene preceeded by a polylinker and followed by the SV40 polyadenylation site. Subclone B90RA20 (+) was obtained by deletion of the PstI/AvaI 355 bp fragment of B9 and re-ligation. The resultant construction contains the 1731 5'LTR, preceeded by a 54 bp Drosophila genomic sequence and followed by an 8 bp internal 1731 sequence. The 422 bp HindIII fragment of subclone B90RA20 was prepared and digested either by DdeI or by BbvI. The 323 bp HindIII/DdeI fragment and the 208 bp HindIII/BbvI fragment were subcloned into the pCAT12 SmaI restriction site (subclones were named D64 (+), D65 (−), B11 (+) and B11.15 (−) respectively). The 777 bp HindIII fragment of subclone B26 was prepared and digested by BbvI. The resultant 236 bp HindIII/BbvI fragment was subcloned into the pCAT12 SmaI restriction site (B2.5: +; B2.6: −). The 4.3 Kb NdeI/NdeI fragment of the pFP5c clone of 1731 (10) was also subcloned into the pCAT12 SmaI restriction site (N42: +). The HindIII/ApaI 319 bp and HindIII/DdeI 235 bp fragments of this subclone were prepared and cloned in the SmaI restriction site of pCAT12 (A88: +; A35: −; D3: +; D45: −respectively). The 1.4 Kb SalI/BstNI fragment of pFP3c (10) which contains the 3'LTR of 1731 preceeded by a 700 bp internal 1731 sequence and followed by 10 bp of *Drosophila* genomic sequences was subcloned into the pCAT12 SmaI restriction site (L3D12: +; L3i5: −). The maps of these subclones are shown on Figure 1.

DNA transfections and CAT assay

Drosophila melanogaster Schneider 2 (S2) cells (22) were seeded at a density of 10^7 cells 24 hours before transfection and then transfected using the DNA-calcium phosphate procedure of Wigler et al. (23). Cells were rinsed with TBS (Tris-buffered saline: 14 mM NaCl, 5 mM KCl, 25 mM Tris-HCl pH 7.4) then buffer was changed with 5ml of fresh medium (Schneider medium supplemented with 5% fetal calf serum) and 1ml of DNA/Ca-

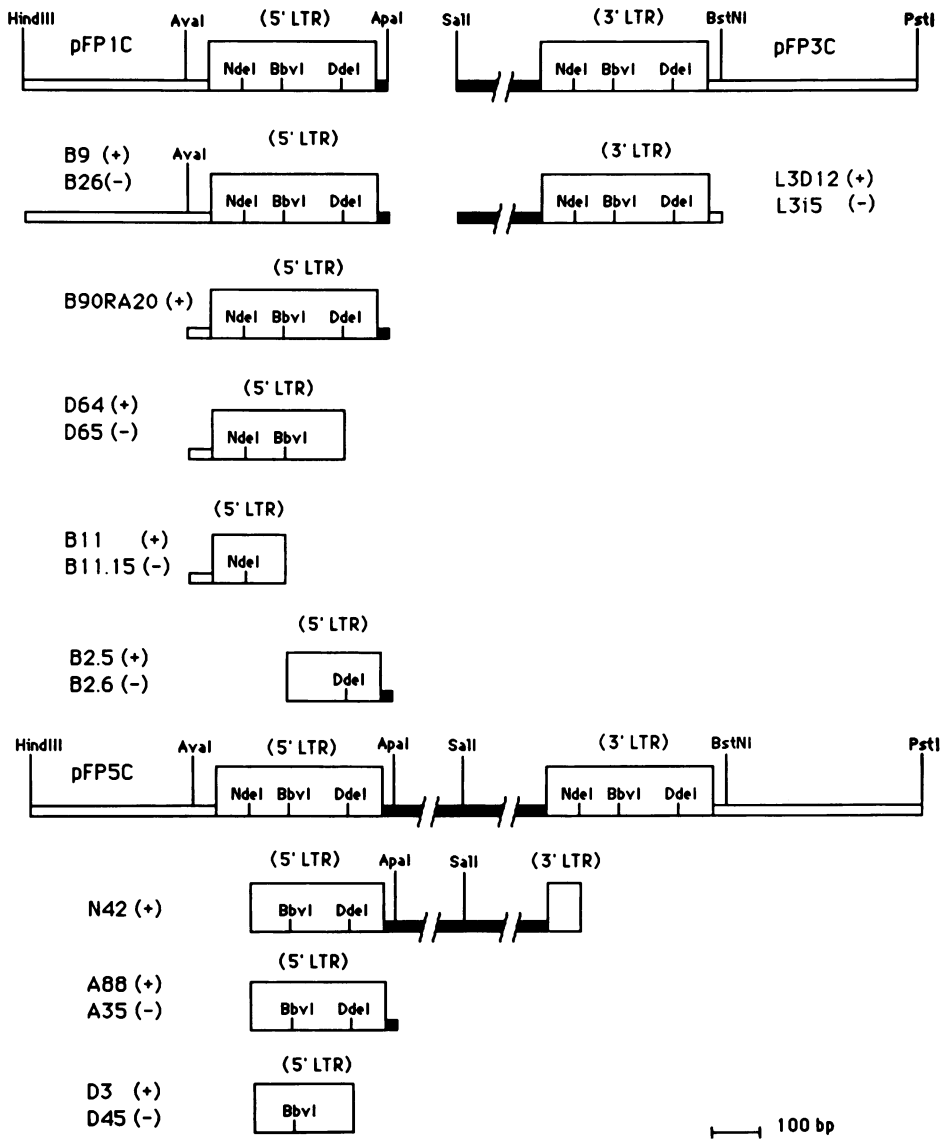


Figure 1: 1731 DNA segments cloned into pCAT12 vector.
Boxes represent the 1731 5′ and 3′ LTRs. Solid bars correspond to 1731 internal sequence and open bars to *Drosophila* flanking sequences. Subclones pFP1c, pFP3c and pFP5c were previously described (10). See Material and Methods for details about other subclones.

phosphate solution (containing 40μg of plasmid DNA) was added. After 4 hours of incubation, cells were treated for 2 minutes with 15% glycerol in TBS buffer. The glycerol mixture was then changed with 3ml of fresh medium. Extracts were prepared 22 or 40 hours after transfection and chloramphenicol acetyl transferase (CAT) assays were

performed as described (24). The enzymatic reaction was stopped after 1, 2 and 3 hours to verify the linear range of conversion of chloramphenicol to its acetylated derivatives. Chloramphenicol and its acetylated forms were separated on thin layer chromatography. After autoradiography, spots were cut out and counted in scintillation fluid.

Hormonal treatment

20-Hydroxyecdysone (20-OH) was added to culture medium at 0.1μM final concentration 22 hours after transfection (i.e. 18 hours before collecting the cells for CAT assays). The hormonal response was checked by measuring the catalase activity in transfected Drosophila S2 cells, 48 hours after addition of 20-OH in the culture medium following the protocol previously described (7).

RNA isolation and primer extension analysis

Total RNAs were extracted as previously described (10) from untransfected cells and from cells transfected with D64 and D3. A synthetic 30-mer complementary to the sense (+) strand of the CAT gene (position 51 to 80) was 5′ end-labelled with ATP-gamma-^{32}P (5000 Ci/mmole) and polynucleotide kinase (Amersham). About 0.2 pmole (10^6 cpm) of this oligonucleotide was annealed to 30μg of RNA in 50% formamide, 40mM Pipes pH 6.4, 0.5M NaCl and 1mM EDTA at 42°C overnight. Primer extension was performed in 50mM Tris.HCl pH8.3, 6mM $MgCl_2$, 40mM KCl, 1mM DTT at 37°C for 30 min in the presence of dATP, dCTP, dGTP and dTTP (50μM each) and 50U of avian myeloblastosis virus reverse transcriptase. The samples were heated at 90°C for 3 min and loaded onto a 6% polyacrylamide, 8M urea sequencing gel.The DNA sequence used as a marker was generated using the above 30-mer as the primer in dideoxy sequencing reactions on the double-stranded D3 template previously denatured in 0.2N NaOH.

RESULTS

Promoter function of the LTRs of 1731

The 1731 LTRs (15) (Figure 2) contain sequences that are known in eukaryotes to be essential for transcriptional regulation. We detected a putative TATA-box in position 110 as well as two putative CAAT-boxes (25) in positions 12 and 40, included in a 28 imperfect direct repetition and in position 75, an octanucleotide present in the Simian virus 40 enhancer element (26). The existence of these transcriptional signals strongly suggests that the 1731 LTRs constitute a promoter. To check this hypothesis, we made constructions where 1731 LTRs were cloned upstream of the CAT reporter gene. In these transient expression assays of transfected plasmid DNA, the measured CAT activity reflects the strength of the promoter.

Promoter function of the 5′LTR

The B9 (+ orientation) and B26 (− orientation) subclones were tested for CAT activity in *Drosophila* cells. This enzymatic activity was measured 22 and 40 hours after transfection. CAT gene expression is only detected when the 5′LTR and the CAT gene are adequately oriented with regard to transcription (subclone B9). The activity reaches a plateau after 22 hours then remains stable up to 40 hours after transfection. When the 1731 LTR and the CAT gene are in opposite orientation (suclone B26), the measured CAT activity is equivalent to the background (i.e. non transfected cells or cells transfected with the pCAT12 vector).

Subclone B90RA20, which contains the 5′LTR of 1731 preceeded by a 54 bp *Drosophila* genomic sequence and followed by 8 bp of a 1731 internal sequence, was also tested for CAT expression. The CAT activity detected is comparable to that of B9 subclone. The

Table 1: Comparison of the promoter activity of the 5′ and 3′ LTRs of 1731 in the sense (+) or antisense (−) orientation.

Time after transfection	22h	40h		
CAT reaction	2h	1h	2h	3h
B9	97.0 ± 16.0 (5)	56.4 ± 3.6 (5)	100.0	191.0 ± 41.2 (5)
B26	12.0 ± 5.6 (3)		14.0 ± 6.5 (6)	
B90RA20	74.9 ± 20.5 (3)		118.5 ± 22.9 (4)	
L3D12	81.6 ± 15.0 (3)	50.0 ± 8.0 (2)	106.5 ± 18.0 (3)	133.6 ± 21.6 (2)
L3i5			3.2 ± 2.1 (4)	
pCAT12			2.0 ± 0.5 (4)	

CAT assays were performed 22 or 40 hours after transfection and CAT reactions were allowed to continue for 1, 2 or 3 hours: percent conversion of chloramphenicol to its acetylated derivatives are shown. The relative CAT activity was calculated by normalizing the percent conversion for subclone B9 equal to 100. Numbers in parenthesis indicate the number of experiments realized for each subclone.

results are given on Table 1. These experiments allow us to localize the promoter function in the 398 nucleotides including the 5′LTR.

Promoter function of the 3′ LTR

The nucleotide sequences of the 1731 5′ and 3′ LTRs show 96% homology (Figure 2). It was thus interesting to compare their promoter function. Subclones L3D12 and L3i5 which contain the 3′LTR of 1731 were tested for CAT expression in *Drosophila* cells. As previously described for the 5′LTR, CAT activity is only detected when the 3′LTR and the CAT gene are in the same orientation with regard to the transcription (subclone L3D12) (Table 1).

Comparison of the 5′ and the 3′ LTR/CAT subclones

Comparison of the 5′ and 3′ LTR subclones are summarized in Table 1. These experiments show that base differences between the 5′ and the 3′LTR do not play a crucial role, neither in the sense, nor in the strength of the promotion. The upstream sequences in the constructions including the 5′ or the 3′LTR being different, we may therefore conclude that the unidirectional promoter function is strictly confined in the LTRs of 1731.

Analysis of deletions in the 5′LTR

To determine the functional importance of the different transcriptional signals previously described, we constructed deletion mutants using the NdeI, BbvI and DdeI restriction sites. The deleted subclones are shown in Figure 1. Each fragment was placed in front of the CAT gene in both the sense (+) and the antisense (−) orientation. CAT activity in *Drosophila* transfected cells was measured and calculated by normalizing the chloramphenicol acetylation for subclone B9 equal to 100. The enzymatic activity of all the antisense constructions (D65, B11.15, B2.6, A35 and D45) is at the level of the background, confirming the unidirectional function of the promoter.

Deletion of the last 76 nucleotides of the 5′LTR (subclone D64) results in a four fold increase of the measured CAT activity. Moreover,subclone B11, which corresponds to the deletion of the last 191 nucleotides of the LTR, is inactive in promoting CAT expression. This last deletion begins 29 nucleotides downstream of the putative TATA-box. The complementary subclone (B2.5) (i.e. the subclone which contains the last 195 nucleotides

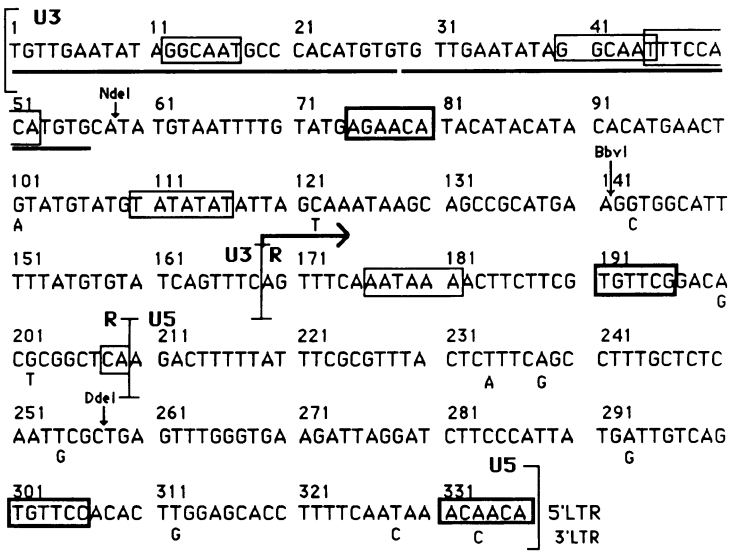


Figure 2: Nucleotide sequence of 1731 LTRs (10).
Sequence of the 5′ LTR is shown on the top line. Positions of divergence with the 3′ LTR are show below each line. Direct repeats are underlined. The putative control sequences are framed: positions 12 and 40: CAAT-boxes; position 45: octanucleotide of the SV40 enhancer type (26); position 110: TATA-box; position 176: polyadenylation signal; position 208: putative polyadenylation site; positions 75, 191, 301 and 331: sequences similar to the hexanucleotide involved in the binding of the glucocorticoid receptor on MMTV LTRs (16,17). The arrow indicates the initiation of transcription.

of the 5′ LTR) looses the promoter activity. Deletion of the first 58 nucleotides of the 5′LTR (A88) results in a five fold decrease in promoter strength. Deletion of both the first 58 and the last 76 nucleotides of the 5′LTR (D3) results in a 1.6 fold increase of the enzymatic activity. Analysis of subclone N42, which amounts to testing the first 60 nucleotides of the LTRs, shows that this segment is inactive in CAT expression promotion. The results are summarized in Figure 3 and Table 2. All these subclones can be ranked with regards to their promoter strength D64 > D3 > B9 > A88 > B11, B2.5, N42. This suggests that the 1731 LTRs could be divided into three fuctional domains. The central domain (nucleotides 59 to 257) contains the core promoter. The last 76 nucleotides could represent a second domain which might be a silencer. The third domain (the first 58 nucleotides of the LTR) located at the 5′ end of the core promoter seems to play an activator role (see also discussion).

Localizations of the hormone regulatory sequences in the 1731 LTRs

The 1731 polyA+ transcripts decrease under 20-OH treatment (10, J.Becker, in preparation). The earliness of the hormonal response is suggestive and consistent with a direct effect of 20-OH at the transcriptional level. The existence in the 1731 LTRs of an hexanucleotide 5′-AGAACA-3′ (position 75) identical to the hexanucleotide involved in the binding of glucocorticoid and progesterone receptors to MMTV LTRs (16, 17, 27) was previously described (10, Figure 2). Three nearly identical hexanucleotides exist in positions 191, 301 and 331 of the 5′LTR. It was thus interesting to check the steroid hormone action on CAT activity for the different subclones described here.

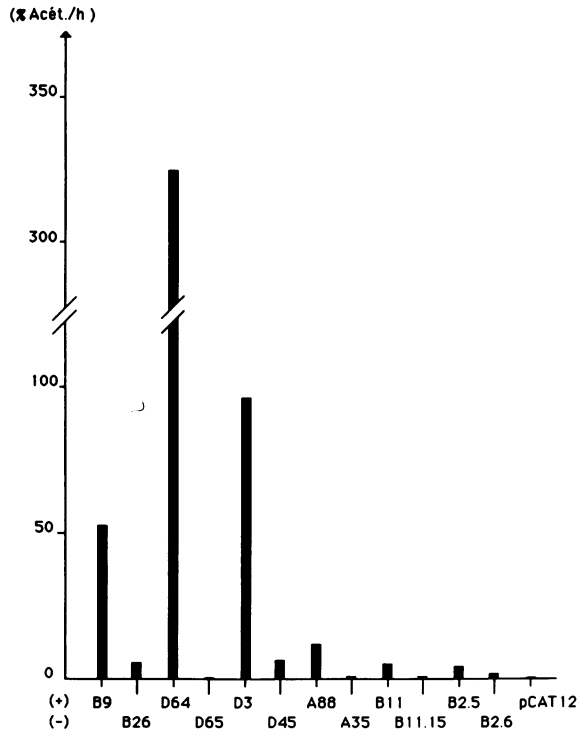


Figure 3: Comparison of 1731 LTR/CAT deletion subclones. (see Table 2)
The percent conversion of chloramphenicol to its acetylated forms per hour is shown in ordinate.

Drosophila cells were transfected with B9 subclone DNA and 20-hydroxyecdysone was added to the culture medium 22, 38 and 39 hours after transfection (i.e. 18, 2 and 1 hours before collecting the cells). The results are shown in Table 3. Decrease of CAT activity is detectable as soon as 2 hours after hormonal treatment and drops at least by a factor of two after 18 hours of treatment. In order to verify the hormonal effect on the *Drosophila*

Table 2: Comparison of 1731 5′ LTR/CAT subclones in both sense (+) and antisense (−) orientations.

Hormonal treatment	Relative activity
None	100.0
1h	85.5 ± 9.2 (3)
2h	42.5 ± 16.2 (6)
18h	43.0 ± 16.5 (20)

CAT assays were performed 40 hours after transfection and CAT reactions were allowed to continue for 1, 2 and 3 hours. Relative CAT activity was calculated by normalizing the percent acetylation of chloramphenicol to its acetylated derivatives to subclone B9. Numbers in parenthesis indicate the number of experiments realized for each clone.

Table 3: Effect of hormonal treatment on subclone B9.

CAT reaction	1h	2h	3h
B9	56.4 ± 3.6 (5)	100.0	191.0 ± 41.2 (5)
B26		14.0 ± 6.5 (6)	
D64	355.6 ± 47.0 (3)	667.2 ± 85.1 (3)	853.5 ± 110.3 (3)
D65		2.8 ± 1.2 (4)	
D3	127.0 ± 28.8 (4)	167.5 ± 55.7 (6)	271.8 ± 36.6 (4)
D45		12.2 ± 4.0 (2)	
A88	11.4 ± 6.5 (6)	26.1 ± 11.8 (6)	30.6 ± 19.0 (6)
A35		3.1 ± 2.3 (5)	
B11		7.1 ± 4.1 (5)	
B11.15		2.3 (1)	
B2.5		10.2 ± 5.8 (4)	
B2.6		3.8 ± 1.4 (3)	
N42		0.9 (1)	

20-OH was added to culture medium at 0.1μM final concentration 1,2 or 18 hours before preparation of the extracts. Percent conversion of chloramphenicol to its acetylated derivatives is expressed by normalizing the results obtained with untreated transfected cells equal to 100.

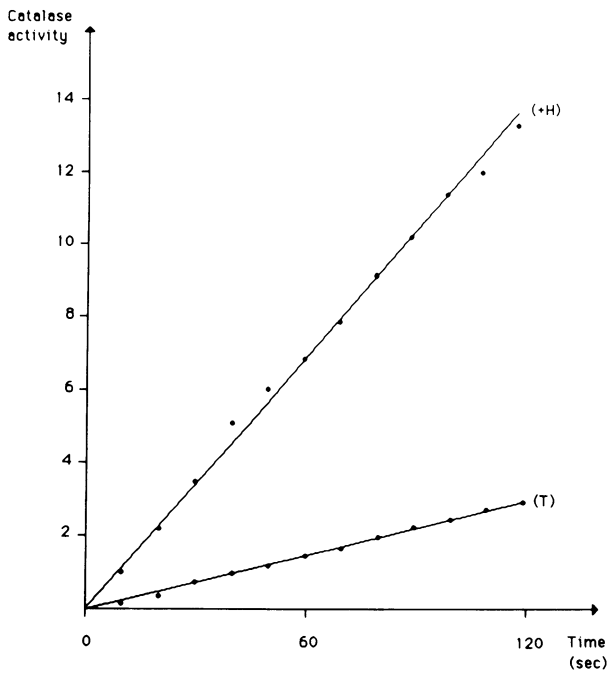


Figure 4: Hormonal induction of catalase activity in B9 transfected cells.
The results are represented on a semi-logarithmic plot (see ref. 7 for details). Catalase activity was measured 48 hours after addition of 20-OH in the culture medium of cells transfected with subclone B9. T: untreated control cells; H: Hormone treated cells.

Table 4: Effect of 20-OH treatment on subclones L3D12, D64, D3 and A88.

Hormonal treatment	None	18h		
CAT reaction	2h	1h	2h	3h
B9	100.0	40.9 ± 8.4 (5)	43.0 ± 16.5 (20)	105.8 ± 18.2 (5)
L3D12	106.5 ± 18.0 (3)	10.7 ± 2.5 (3)	23.4 ± 5.7 (3)	35.9 ± 14.0 (3)
D64	667.2 ± 85.1 (3)	114.3 ± 37.1 (4)	175.4 ± 61.4 (6)	231.8 ± 50.8 (4)
D3	167.5 ± 55.7 (6)	131.8 ± 12.8 (5)	214.1 ± 42.6 (10)	271.7 ± 54.9 (5)
A88	26.1 ± 11.8 (6)	26.7 ± 8.3 (2)	34.5 ± 11.4 (4)	45.4 ± 14.1 (2)

20-OH was added to culture medium at 0.1μM final concentration 18 hours before preparation of the extracts. Percent conversion of chloramphenicol to its acetylated derivatives is expressed by normalizing the results obtained for cells transfected with subclone B9 in absence of 20-OH equal to 100. Numbers in parenthesis indicate the number of experiments realized for each clone.

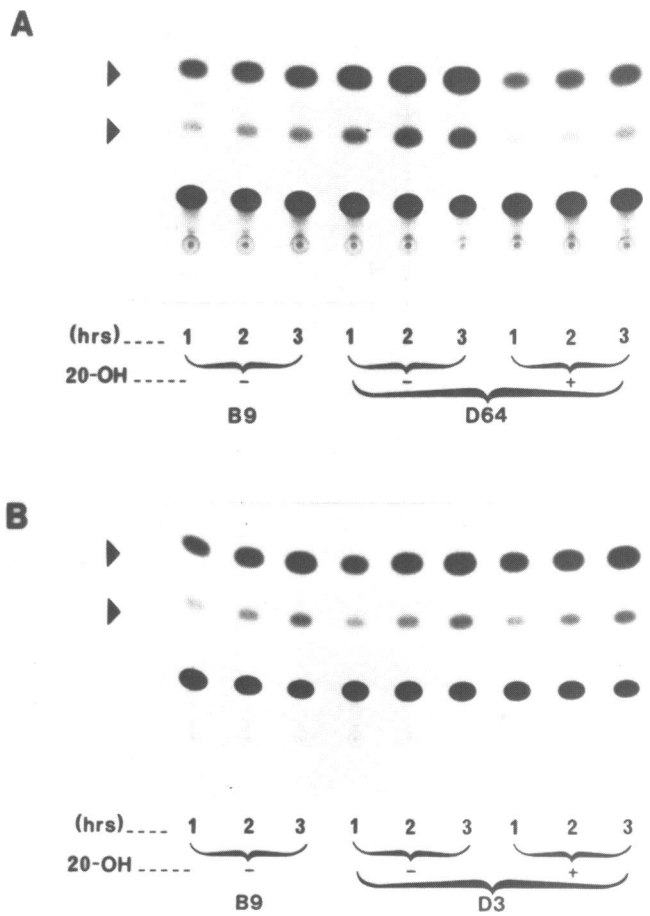
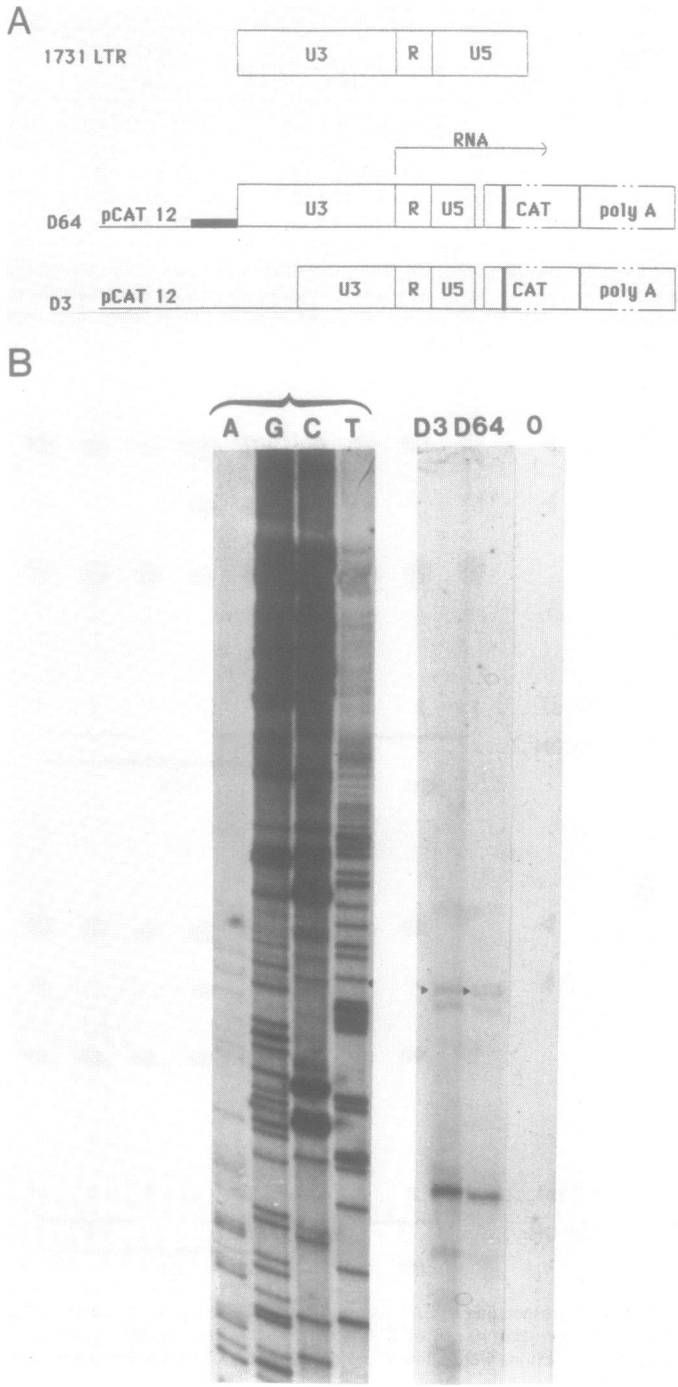


Figure 5: Autoradiogram corresponding to CAT assays from a representative transfection of D64 (A) with (+) or without (−) hormonal treatment (10 μM of 20-OH during 18 hours) and D3 (B) with (+) or without (−) hormonal treatment. CAT reactions were allowed to continue for 1, 2 or 3 hours (respectively 1, 2 and 3) in order to verify the linear range of the reaction.


A
1731 LTR
U3
R
U5
RNA
D64
pCAT 12
U3
R
U5
CAT
poly A
D3
pCAT 12
U3
R
U5
CAT
poly A
B
A G C T
D3 D64 0

S2 cells, the catalase activity was measured in cells transfected with subclone B9, 48 hours after addition of 20-OH in the culture medium. The results are shown in Figure 4 and reveal a 5 fold increase of catalase activity in the treated cells.

Table 4 shows the CAT activity measured after 18 hours hormonal treatment for the subclones L3D12, D64, D3 and A88. The enzymatic activity remains undetectable after hormonal treatment of cells transfected with antisense plasmids (i.e. L3i5, D65, D45 and A35) (data not shown). Comparison of subclones B9 (5'LTR subclone) and L3D12 (3'LTR subclone) indicates that the LTRs should contain the hormone regulatory sequences (Table 4). The hormonal effect is maintained after deletion of the 76 nucleotides downstream of the 5'LTR (subclone D64) (Figure 5). Taken together, these results allow us to localize the hormonal regulatory sequences in the first 260 bp of the LTR. Moreover, the hormonal effect is lost with deletion of the 58 first bp of the LTR (subclone D3) (Figure 5) which corresponds to the lost of the 2 putative CAAT-boxes and of the octanucleotide of the Simian virus 40 enhancer type (Figure 2).

In order to confirm that changes in CAT expression are due to changes in the level of transcription of the vectors, the initiation of transcription was determined for subclones D64 and D3 by primer extension experiments (Figure 6). A complementary oligonucleotide corresponding to position 51 to 80 of the CAT gene was hybridized with RNAs extracted from D64 or D3 transformed cells and extended with AMV reverse transcriptase. The results obtained with the two subclones are identical. Comparisons with dideoxysequencing reactions performed with the D3 subclone (the 3' ends of the insert are the same for the two subclones) allow us to locate three transcription starts at positions 168, 173 and 212 of the 5'LTR (see Figure 2) in the two subclones. The most upstream initiation site corresponds to the normal start of 1731 transcription in *Drosophila* cells (data not shown).

DISCUSSION

Our results demonstrate the existence of a unidirectional promoter in the 336 bp which constitute the 1731 LTRs. This is consistent with our previous data showing the unidirectional sense of 1731 transcription (15). Expression of the reporter CAT gene is obtained not only with the 5'LTR but also with the 3'LTR as was described in the case of the Mouse Intracisternal type A Particles (28). The fact that the 3'LTR of a retrotransposon is able to promote the expression of a flanking gene is important, considering that these retroviral-like elements are mobile genes. Indeed, insertion in a new genomic locus might introduce a new promoter, therefore provoking inappropriate expression of an adjacent gene. It is now evident that such mobile genetic elements could activate the expression of cellular proto-oncogenes as already shown for vertebrate retroviruses (29). Deletion analysis of LTR/CAT fusion genes indicates that sequences downstream of position 260 are not essential to obtain expression of the reporter gene. The increase of CAT expression together with the loss of the last 76 nucleotides (D64 compare to B9) could

Figure 6: A) Schematic representation of subclones D64 and D3. The black box in the CAT gene shows the synthetic oligonuclotide used in the primer extension experiments. Thick lines indicate the 5'LTR flanking sequences. B) Primer extension mapping of the transcription inititiation sites in the D64 and D3 subclones. RNAs isolated from untransformed cells (line 0) or from cells that had been transformed with D64 and D3 were hybridized with the single-end-labelled 30-mer complementary to the CAT gene (position 51 to 80) as described in Material and Methods.
A, G, C, T, dideoxysequencing reactions performed with the same oligonucleotide on the D3 double-stranded template. The arrows indicate the band which corresponds to the normal initiation start of 1731 in *Drosophila* cells.

Figure 7: Homologies between 1731 and the DNA targets for Octa1-c and HIV.NFkB :—Octa1-c, a POU homoeoprotein (37). — HIV. NFkB, a Nuclear transcriptional Factor which binds to nearly identical sequences (AGGGACTTTCC and GGGGACTTTCC) found in the U3 part of the human immunodeficiency virus type I (HIV I) (38 and references therein).
Number in parenthesis indicates the position of the sequence in the LTR (see Figure 2).

be interpreted as an effect of decreasing distance between the promoter and the reporter gene. Nevertheless, we cannot exclude the existence of a silencer located in these 76 nucleotides as described in the case of the MMTV promoter (30). The fact that subclone B11 is inactive allows us to delimit the functional region of the promoter. Finally, removing the first 58 nucleotides (subclone D3 compare to D64) results in a decrease of CAT activity. The transcription starts of these subclones are the same, so the structure and therefore the stability of the corresponding CAT RNAs are comparable and the CAT activity directly reflects the strength of their promoter. This observation can then be explained by the existence of an activator in this region. This last point has to be related to the existence of transcriptional regulatory signals in these 58 nucleotides (putative CAAT-boxes and octanucleotide of the Simian virus 40 enhancer type). The loss of both the putative activator and silencer is consistent with the relative CAT activity observed with subclone D3 (Figure 3 and Figure 5).

We also show here that 1731 LTRs can confer 20-OH responsiveness to the reporter gene. The observed inhibitory effect is not a general feature since an endogenous enzymatic activity (catalase) is induced by the hormone at the same time. The analysis of genes inducible by steroid hormones in Vertebrates has shown that steroid receptors bind discrete DNA segments in vitro. Gene-transfer experiments have revealed that these segments are required for hormone induction in vivo (for reviews see 31, 32). The consensus glucocorticoid receptor binding sequence was found to be 5′-(G/C)gGt(A/T)CANNTGT (C/T)CT-3′ with the hexamer TGT(C/T)CT particularly well conserved (33, 34). This consensus sequence was established from several inducible genes, including a *Drosophila melanogaster* ecdysone inducible gene (DSC7). The existence of a similar hexamer in 1731 LTRs (in position 75) (on the noncoding strand) suggests a direct action of the hormonal receptor in this region. Moreover, sets of conserved hexanucleotides exist in the LTRs of Mouse IAPs and of the *Drosophila melanogaster* retrotransposon 412 (35,10). IAP transcription is induced by the steroid hormone dexamethasone (35) whereas 412 transcription is repressed by 20-hydroxyecdysone (36) reinforcing the hypothesis that the mentioned hexanucleotides take part in the steroid regulation of these retrotransposons. Deletion of the 76 nucleotides downstream of the 1731 LTR conserves the hormonal response, suggesting that the two imperfect nucleotides in position 301 and 331 are not implicated. A preliminary experiment of methylation protection after binding of the glucocorticoid receptor on the 1731 LTR shows protection of the G at position 76, which corresponds to the conserved hexamer (E.Slater, personal communication). This last point also argues for an effective binding of the 20-OH receptor in this region. However, deletion of the first 58 nucleotides of the 5′LTR provokes the loss of the hormonal responsiveness indicating that, if implicated, the consensus hexameric sequence is not

sufficient for the negative hormonal modulation observed. Other sequences, located in these 58 nucleotides would be necessary to enable the reporter gene to respond to 20-OH. One can thus hypothesize that the observed repression would correspond to the additive or synergistic interaction of different trans-acting factors. Amongst these factors, we can speculate that the hormonal receptor itself binds to the hexanucleotide target while, in its vicinity, other trans-acting factors such as those suggested by target similarities (e.g. Octa1-c or NFkB, see Figure 7) might play (ant)agonist roles.

ACKNOWLEDGEMENTS

Plasmid pCAT12 was a gift of Dr.J.N.Freund, LGEM, Strasbourg). The authors wish to thank Dr.D.Lawrence for helpful discussion and D.Rouillé for excellent technical assistance. This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS) La Fondation pour la Recherche Médicale and l'Association pour la Recherche contre le Cancer (ARC). P.Z. is an ARC fellowship.

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