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A short 5′ region of the long terminal repeat is required for regulation by hormone and heat shock of *Drosophila* retrotransposon 1731

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ABSTRACT

1731, a Drosophila retrotransposon was first described as having a transcription activity which was negatively regulated by 20-hydroxyecdysone (20-OH), the steroid molting hormone of insects. Using constructions expressing the bacterial chloramphenicol-acetyl-transferase (CAT) gene under the control of the entire or deleted Long Terminal Repeats (LTRs) of 1731, we were able to show that a short (28 bp) sequence located in the U3 region of these LTRs was required for 1) the increase in promoter strength, 2) negative regulation by 20-OH and, 3) positive regulation by heat shock.

INTRODUCTION

Embryonic *Drosophila melanogaster* cells cultured in vitro were observed to be responsive to 20-hydroxyecdysone (20-OH), a steroid hormone which plays a crucial role in the cellular differentiation and development of insects. This property was demonstrated by a wide variety of responses such as the hormonal induction of polypeptides (1,2) and enzymatic activities (e.g. acetylcholinesterase (3,4), βgalactosidase (5), catalase (6) reviewed in 7). This allowed us to characterise 1731, a Drosophila melanogaster retrotransposon, the expression of which is negatively regulated by 20-OH (8).

Drosophila cells were also observed to be responsive to heat shock stress. The response included the transcriptional activation of 'heat shock' genes by a trans-acting factor. The 'heat shock factor' (HSF) binds specifically to a 'heat shock element' (HSE) in the promoter of the heat shock genes (reviewed in 9–11).

Retrotransposons are mutagenic, mobile genetic elements which are integrated in the genome of all eucaryotes examined so far. They are similar in appearance to the retrovirus structurally as well as dynamically although their retrovirus-like cycle remains intracellular (reviewed in 12–15).

1731 is moderately repeated in the genome of the *Drosophila melanogaster* cells cultured in vitro as well as in that of the fly. Flanked by two short (5bp) direct repeats it is 4648 bp long formed by two Long Terminal Repeats (LTRs) of 336 bp having a U3-R-U5 structure which frames an internal sequence including a Methionyl-tRNA-binding site and a polypurine rich track at its 5′ or 3′ end respectively, a gag gene and a pol gene. The unidirectional transcription of 1731 generates a major full length polyadenylated RNA whose level decreases following 20-hydroxyecdysone treatment (8, 16, 17). Transfection of constructions promoting bacterial chloramphenicol-acetyl-transferase (CAT) gene expression by diverse fragments of 1731 led, (with regard to the transcriptional mechanism of the steroid action), to the following data (18). A steroid non regulable core promoter is located in the 5′ LTR. This core promoter is flanked at its 3′ end by a U5 negative regulatory sequence which is not required for hormonal regulation. It is flanked at its 5′ end by a 121 bp fragment (including a genomic extra-LTR 63 bp long sequence and the first 58 base pairs of the U3 region) which has a positive regulatory function and which is required for steroid regulation.

We show here that a 28 bp long fragment included in the first 58 base pairs of the U3 region is required for a 50-fold increase in the promoter strength, for the negative regulation by the steroid and for the positive regulation by heat shock. It is important to emphasize that such studies concerning the regulation of the expression of a retrotransposon could be linked to its rate of transposition and thus to the probability of provoking mutations.

MATERIALS AND METHODS

Construction of plasmids

The 121 bp long fragment of D64 plasmid (18 and figures 1 and 2), which contains the 63 bp of genomic sequences located upstream from the 5′ LTR plus the first 58 bp of the U3 region, was replaced (vectors L1 and L2) by a synthetic oligonucleotide, an exact copy of the beginning of the LTR. (ie between nucleotide −168 and the NdeI restriction site, figures 1 and 2). This oligonuceotide, referred to as oligonucleotide 1 posseses a 5′ end which is compatible with the NdeI restriction site and a 3′ end compatible with the PstI restriction site. The 371 bp HindIII fragment of plasmid D64 was subcloned in the HindIII digested Bluescript SK+ vector (Stratagene), which does not contain any NdeI restriction site. The BS14 plasmid was thus obtained. The oligonucleotide 1 was cloned as described by Mounts et al (19) between the PstI and NdeI restriction sites of BS14 to obtain BS1. The XbaI-HindIII fragment of BS1 was then prepared, repaired with dXTP and T4 DNA polymerase and then cloned into the

SmaI restriction site of the pCAT12 vector (18). We thus obtained subclone L1 (sense orientation with regard to CAT gene) and L1(−) (antisense orientation). These L1 clones are identical to the D64 ones apart from the major difference that they do not possess any Drosophila genomic sequence upstream from the LTR (figures 1 and 2).

The beginning of the 1731 LTR U3 region comprises two almost perfect direct repeats (26/28, figure 2). This fragment was replaced by a synthetic oligonucleotide (referred to as oligonucleotide 2) including the proximal repeat with regard to the TATA box. It possesses a 5′ end compatible with the NdeI restriction site and a 3′ end compatible with the PstI restriction site. The oligonucleotide 2 was thus cloned according to Mounts et al (19) between the PstI and NdeI restriction sites of BS14 (figure 1) giving BS2 and finally L2 and L2(−). The L2 clones are about identical to the L1 ones, the major difference being that they only possess the proximal repeat of the beginning of the U3 region in the 1731 LTR. The other plasmids used in this study: B90RA20 (containing the 336 bp long 5′ LTR flanked at the 5′ and 3′ ends by extra LTR sequences), L3D12 (containing the 336 bp long 3′ LTR, also flanked at both ends by extra LTR sequences), D3 (referred to as the core promoter: figure 2) have already been described (18). D64, L1 and L2 also contain bacterial chloramphenicol-acetyl-transferase (CAT) reporter gene.

HSP-CAT1 plasmid (20), (generous gift from Dr I.B. Dawid), contains the heat shock inducible promoter of the Drosophila 70 heat shock gene which directs the CAT gene.

Drosophila cell transfection

The *Drosophila melanogaster* S2 cells (21) were seeded at a density of 10^7 cells/flask 24 hours before transfection, at 18°C. Transfection was carried out using the DNA calcium phosphate procedure of Wigler et al (22). The cells were washed. 40 hours after the transfection, the extracts were prepared and the CAT assays performed as previously described (18). CAT reactions were allowed to proceed for 1, 2 and 3 hours to show that activities were measured in the linear range. The conversion of chloramphenicol to its acetylated derivatives given as a percentage is expressed by normalizing the results obtained with cells transfected with subclone D64 DNA in the absence of 20-OH.

Hormonal and heat shock treatment

Treatment with 20-hydroxyecdysone (20-OH) was performed 22 hours after tranfection at a final concentration of 0.1 μM. Heat shock was carried out at 37°C for 1 to 5 hours, 40 hours after transfection. The S2 response to heat shock was checked by transfecting S2 cells with the HSP-CAT1 plasmid.

RESULTS

Promoter function and deletions in the U3 region of the 1731 5′ LTR: a combination of enhancement and silencer effects

The 121 bp long fragment, located between HindIII and the NdeI restriction sites at the 5′ end of the D64 vector (figure 2), displays a positive regulatory function. This fragment contains 63 nucleotides of genomic sequences fused to the first 58 nucleotides of the LTR U3 region. In order to determine the influence of the flanking genomic sequences we compared the CAT activity expressed by the clone D64 which possess these genomic sequences with that of L1 which does not.

The results (table 1) show that the positive regulation effect

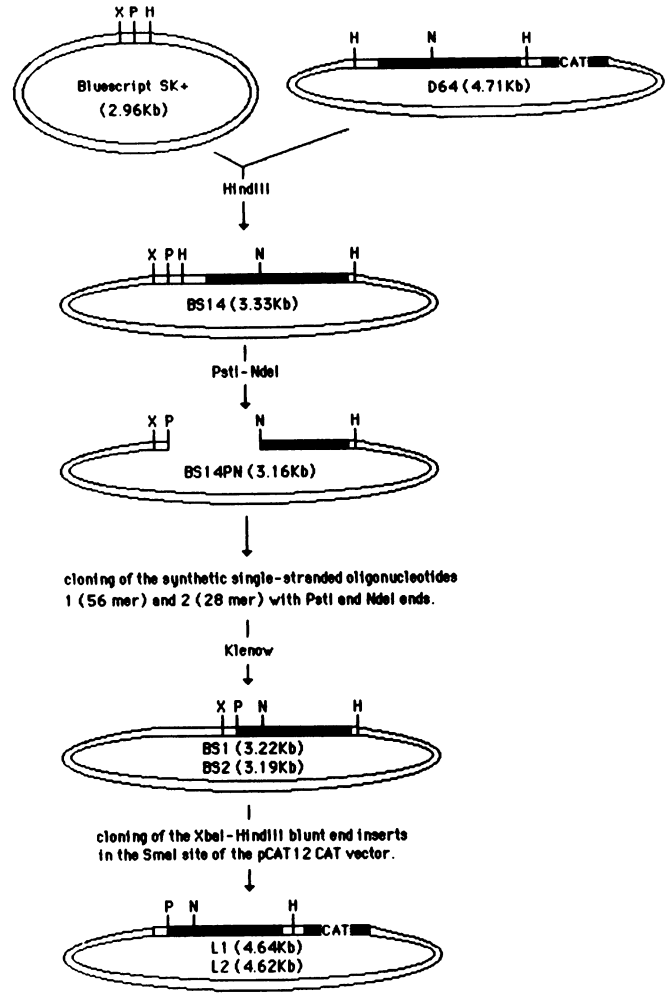

Figure 1. Construction of plasmids containing the modified 5′ LTR of 1731 upstream from the reporter CAT gene (see Materials and methods).

is due to the 121 bp fragment (D64/D3 = 4). This effect is not due to the upstream genomic sequences (L1/D64 = 1), but is due to the first 58 bp of the U3 region of the LTR (L1/D3 =4). Note that the low CAT activity obtained with L1(−) is consistent with the unidirectional transcription of 1731 (16).

The 58 bp beginning the LTR includes two almost perfect direct repeats (figure 2). We wanted to establish whether this repetition was necessary for the positive regulation effect. The proximal repeat was sufficient to multiply the CAT activity by at least 50-fold (L2/D3 = 55). When the two repeats act together, the distal one (which begins the LTR) diplays a negative modulator function (L1 compared to L2: L1/L2 = 0.07). We concluded that the positive regulation effect of the first 58 bp of the U3 region is the combination of the powerfull (50-fold) activator role of the proximal repeat associated to the smaller negative modulator located in the distal repeat.

A 28 bp long fragment coinciding with the proximal repeat of the U3 region of the LTR is required for the negative regulation of 1731 by 20 hydroxyecdysone

The HindIII-NdeI 121 bp long fragment of D64 not only increases CAT expression in D64 construction but is also required for the

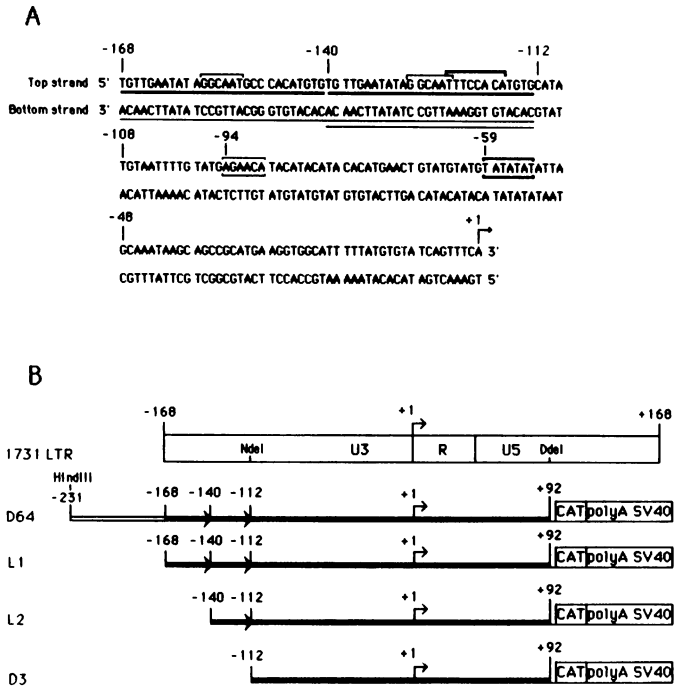

Figure 2. The 1731 5' LTR U3 region nucleotidic sequence and schematic representation of subclones. (A), nucleotide sequence of the 5' LTR U3 region. On the top strand; positions −157 and −129 are shown: CAAT boxes; position −124: octanucleotide of the SV40 enhancer type; position −94: sequence identical to the hexanucleotide (GRE) involved in the binding of the glucocorticoide receptor; position −59: TATA box. Direct repeats (−168,−141;−140,−113) are underlined. On the bottom strand sequences are underlined which correspond to the synthetic oligonucleotides (Pharmacia) used: (−168,−113), oligonucleotide 1; (−140,−113), oligonucleotide 2. (B), schematic representation of subclones with partial deletions of the 5' LTR U3 sequence (63 bp of Drosophila genomic fragment upstream from the LTR in D64 are in white whereas the LTRs sequences are in black.

Table 1. Promoter activity of the 5'LTR/CAT subclones in both sense and antisense (−) orientation.

CAT reaction	1h	2h	3h
D64	53.2 ± 7.0 (3)	100	127.9 ± 16.5 (3)
D3	19.0 ± 4.3 (4)	25.1 ± 8.3 (6)	40.7 ± 5.4 (4)
L1	54.6 ± 11.4 (8)	106.1 ± 13.6 (8)	167.4 ± 35.5 (8)
L1(-)	5.5 ± 1.0 (2)	8.0 ± 2.6 (2)	10.6 ± 1.9 (2)
L2	703.7 ± 19.5 (4)	1468.4 ± 184.7 (9)	2906.0 ± 605.0 (4)
L2(-)	5.7 ± 0.9 (2)	9.9 ± 1.6 (2)	18.0 ± 2.5 (2)

CAT assays were performed 40 hours after transfection and CAT reactions were allowed to continue for 1, 2 and 3 hours respectively. Relative CAT activity was calculated by normalizing the percent acetylation of chloramphenicol to its acetylated derivatives to subclone D64. Numbers in brackets indicate the number of experiments carried out for each clone.

negative regulation by the steroid hormone 20-hydroxyecdysone. Its deletion leads to the D3 construction referred to here as the core promoter which has lost the property to be regulated hormonally (18). Comparisons of the CAT activities expressed by the D64, D3, L1 and L2 constructions in the absence and in the presence of 20-hydroxyecdysone (table 2) show that deletion of the 121 bp long fragment abolishes the negative regulation by the steroid (D64 vs D3) and demonstrate that

Table 2. Effect of 20-hydroxyecdysone treatment on subclones D64, D3, L1 and L2.

Hormonal treatment	−	+	
CAT reaction	2h	1h	2h	3h
D64	100	17.1 ± 5.5 (4)	26.2 ± 9.2 (6)	34.7 ± 7.6 (4)
D3	25.1 ± 8.3 (6)	19.7 ± 1.9 (5)	32.0 ± 6.3 (10)	40.7 ± 8.2 (5)
L1	106.1 ± 13.6 (8)	5.5 ± 2.1 (3)	10.8 ± 3.9 (5)	22.7 ± 5.5 (3)
L2	1468.4 ± 184.7 (9)	77.2 ± 34.0 (2)	138.4 ± 26.8 (2)	190.1 ± 35.3 (2)

The hormone was added to the culture medium at a final concentration of 0.1 μM 18 hours before preparation of the extracts. CAT assays were performed 40 hours following transfection and CAT reactions were allowed to continue for 1, 2 and 3 hours respectively. Percent conversion of chloramphenicol to its acetylated derivatives is expressed by normalizing the results obtained with cells transfected with subclone D64 DNA in the absence of 20-OH. Numbers in brackets indicate the number of experiments carried out for each clone.

Table 3. Similarities between the Drosophila heat shock consensus sequence and the 1731 5'LTR U3 region.

position in the 5'LTR U3 region	Heat shock element (HSE) 5' CTGGAATNTTCTAG 3'	similarity
(-167)	5' GTTGAATATAGGCA 3'	7/14
(-146)	5' CATGTGTGTTGAAT 3'	7/14
(-139)	5' GTTGAATATAGGCA 3'	7/14
(-109)	5' ATGTAATTTTGTAT 3'	10/14
(-96)	5' TGAGAACATACATA 3'	8/14
(-76)	5' CATGAACTGTATGT 3'	7/14
(-26)	5' GTGGCATTTTTATG 3'	9/14

Numbers between brackets indicate the position of the sequence in the LTR U3 region.

hormone repression is only due to the first 58 bp of the LTR U3 region (L1 vs D3). The negative regulation has a factor of 10 (L1 with hormone/L1 control = 0.1). Table 2 shows that the 28 bp long sequence coinciding with the proximal repeat is the only one required for the negative steroid regulation (L2 vs L1) which here again has a factor of 10 (L2 with hormone/L2 control = 0.1). We concluded that this 28 bp long fragment located in the U3 region of the 1731 LTR (position −140 to −113, figure 2) is required for hormone negative regulation as well as for the positive regulation effect.

The 28bp long fragment coinciding with the proximal repeat of the U3 region of the LTR is also required for the positive regulation of 1731 by heat shock

Sequences of the U3 region of the 1731 LTR display similarities with the heat shock regulatory element (HSE) which is known to be required for the gene response to heat shock (10, 11 and table 3). This prompted us to measure the level of CAT expression directed by the entire or partially deleted LTRs during heat shock. It was checked that the CAT expression in clones containing the complete 5' or 3' LTR (respectively constructions B90RA20 and L3D12) was increased by heat shock (data not shown). Comparing the sequence of these two constructions leads

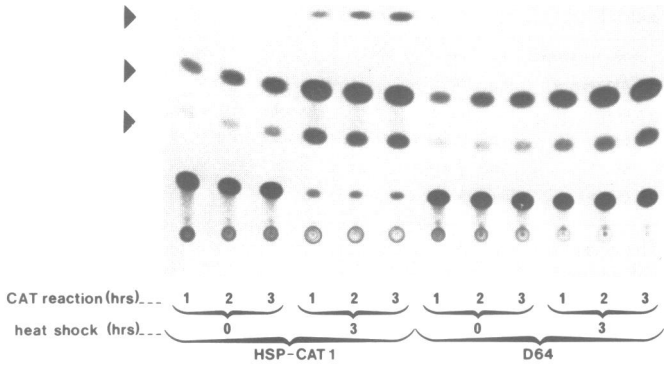

Figure 3. Autoradiogram corresponding to CAT assays from a representative transfection of HSP-CAT1 and D64 with (+) or without (−) heat shock (3 hours at 37°C). CAT reactions were allowed to continue for 1, 2 and 3 hours respectively in order to verify the linear range of the reaction.

Table 4. Effect of heat shock on D64, D3, L1 and L2 subclones. The cells were subjected to heat shock for 4 hours at 37°C.

		D64	D3	L1	L2
CAT activity	Control	1	0.3 ± 0.1 (3)	1.2 ± 0.4 (4)	15.3 ± 4.4 (4)
	Heat shock	6.9 ± 0.8 (4)	0.2 ± 0.1 (3)	13.4 ± 2.1 (4)	80.0 ± 14.0 (4)

Percent conversion of chloramphenicol to its acetylated derivatives per hour is expressed by normalizing the results obtained for cells transfected with D64 subclone in the absence of heat shock. Numbers in brackets indicate the number of experiments carried out for each clone.

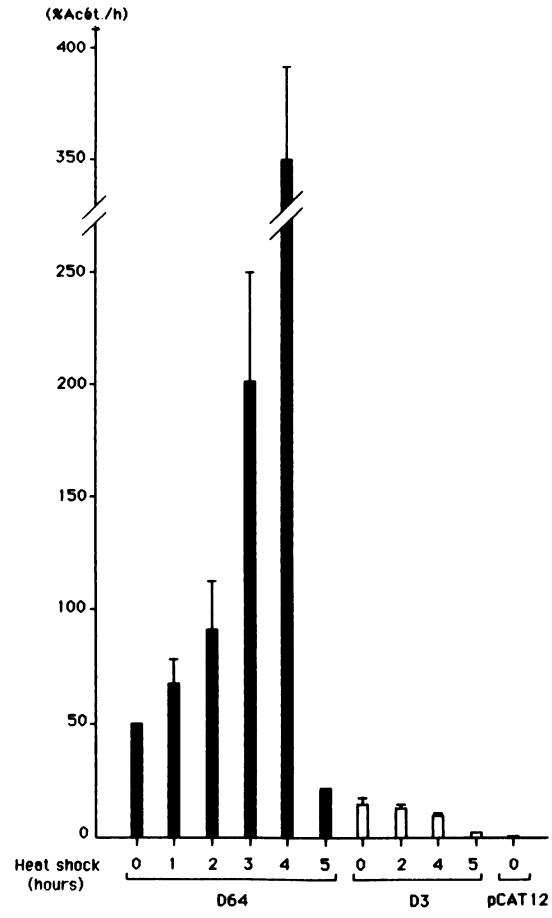

Figure 4. Time course of CAT activity following heat shock in D64 and D3 transfected cells. Cells were subjected to heat shock at 37°C for various time periods between 35 and 40 hours following transfection. They were assayed for CAT activity as described in Materials and methods. Percent conversion per hour of chloramphenicol to its acetylated forms is shown in ordinate. Relative CAT activity was calculated by normalizing the results obtained for cells transfected with D64 subclone in the absence of heat shock.

to the hypothesis that the heat shock regulated promoter should be located somewhere in the 336 bp long 1731 LTRs. This indeed seems to be the case when one observes (figure 3) that CAT expression of D64 is increased by heat shock (about 4-fold less than for the HSP-CAT1 vector which was used as a model). This experiment showed that the last 76 nucleotides of the 5′ LTR are unnecessary for heat shock regulation.

Drosophila cells were transfected with D64 or D3 subclones (figure 4) and subjected to heat shock for various periods of time. The increase of CAT activity was 7-fold higher after 4 hours of heat shock in D64 transfected cells. CAT activity was decreased after 5 hours of heat shock (cells appear to be damaged after long periods of heat shock treatment). On the contrary, in D3 transfected cells, CAT activity is not heat shock regulated. Thus, as a preliminary conclusion, we propose that the HindIII-NdeI 121 bp long fragment of D64 located just upstream from the non regulable D3 core promoter (figure 2) is necessary for positive heat shock regulation. In order to discriminate between the participation of the genomic sequences and the first 58 bp of 5′ LTR U3 region which are included in this 121 bp long fragment, we compared (table 4) the CAT activity in D64 or L1 transfected cells without or following heat shock treatment. As the level of the increase of the CAT activity is about the same (D64 heat shock/D64 control = L1 heat shock/L1 control = 7) we concluded that the addition of the first 58 bp of the U3 region to the non regulable D3 core promoter is sufficient for positive heat shock regulation. The heat shock-induced increase of CAT activity is also about the same in L1 and L2 transfected cells (L1 heat shock/L1 control = L2 heat shock/L2 control = 7) (table 4) we concluded that the 28bp fragment, which coincides with the proximal repeat of the U3 region, is required for positive heat shock regulation.

DISCUSSION

As far as we are aware this is the first case showing that a 28 bp long sequence of a LTR U3 region is required for triple function: activation of the expression of a retrotransposon, negative regulation by a steroid hormone and positive regulation by heat shock.

Concerning the promoter strength of 1731, its LTRs begin with a negative modulator: other examples are known (e.g. 23). The originality of 1731 is that this negative modulator forms a first distal direct repeat (in L1, figure 2) almost identical to the proximal one, located just downstream (present in L1 and L2), which, alone, is able to play a powerful (50-fold) activator role, without changing the polarity of the LTR (see L2(−) in table 1). We noted that the 2 base differences between the distal and

the proximal repeats in this region occur within the canonic sequence of the SV40 core enhancer (24) located in the proximal repeat (position −124, −118 in figure 2). This change would moreover, render this region in the distal repeat (position −152, −145 in figure 2) more homologous to a negative element which has already been described upstream from the proximal initiation site of the *Drosophila melanogaster* alcohol dehydrogenase gene (25). The combined action of these two different fragments (namely distal and proximal repeat) which begin the 1731 LTRs enables a global positive modulation of the expression which is not (at least in this case) disturbed by the flanking genomic sequences.

If the level of the steroid or the heat shock regulation was directly proportional to the level of increase in the promoter strength, one would expect these regulations to differ between the two constructions used (i. e. L2 compared to L1); this however, is not the case. Regulatory interferences between the distal and the proximal repeats appear to be excluded as well as the influences of the extra-LTR genomic sequences. Thus the L2 construction is representative of, and adequate, for studying the mechanisms of these two (steroid or heat shock) opposite regulations.

The regulatory activity associated with L2 was obtained simply by the addition of the 28 bp oligonucleotide 2 to the core promoter (D3) which possesses no such regulatory activity. It is thus tempting to speculate that this oligonucleotide 2 encompasses both a negative Ecdysone regulatory element (n-EcRE) and also a heat shock element (HSE). However, we favour another interpretation for the following reasons. Firstly, no obvious similarity with putative n-EcRE or HSE was detected in oligonucleotide 2. On the contrary, an imperfect HSE was found just downstream (position −109, figure 2, table 3 and ref. 8). Moreover, at position −94, an element identical to a GRE (Glucocorticoid Regulatory Element: 5'TGT(T/C)CT3') has already been observed (8) and does not seem to be completely different from an ecdysone regulatory element consensus recently proposed (26) (sequence of 1731 at position −93: 5'GAACATACATA3' and the consensus sequence: 5'(G/T)NTCANTNN(A/C)(A/C)3'). The second reason is more general: regulations appear to be frequently due to the combined effects of different transcription factors (27−30). For example, the heat shock inducibility of a Xenopus gene depends upon the presence of an HSE but also upon that of an upstream CCAAT signal (31). The inducibility by glucocorticoids of designated constructions (32) is linked not only to a GRE but also to upstream targets for different factors. In the case of 1731, it seems plausible that the heat shock factor or the ecdysone receptor (7) could act through 'position −109' or '−94' only if other DNA binding proteins are present in the region covered by oligonucleotide 2. We have indeed characterized such proteins by gel retardation assay (F. Fourcade-Peronnet, S. Simonart and M. Best-Belpomme, unpublished data).

Our findings concerning the regulation of 1731 need to be modified with regard to retrotransposons. Transposition of the yeast Ty (33) or mouse IAP ('Intracisternal A Particle') retrotransposons (34) has been shown to require intermediate RNA and other evidence suggests that it is a general rule among retrotransposons. It appears that the rate of retrotransposition may be correlated with the rate of transcription. Studies on the positive or negative regulations of the retrotransposons via their long terminal repeats (LTRs) are thus a fundamental field which will provide a better understanding of the control of retrotransposition events.

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