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Volume 328, number 1,2, 183–188 FEBS 12822 August 1993
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# Translation and fates of the gag protein of 1731, a *Drosophila melanogaster* retrotransposon

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Received 3 June 1993

An entire copy of 1731, a *Drosophila melanogaster* retrotransposon, was tagged by fusing in frame its putative gag gene with the reporter LacZ sequence. The high transfection efficiency of *Drosophila virilis* cells added to the absence of 1731 in their genome allowed, by combining histochemical staining and immunological detections, the demonstration of the translation of the 1731 gag gene. The gag protein is gathered in virus-like particles. Its occurrence in nuclei is consistent with a nuclear localization signal. The expression of the sense construction was inhibited by cotransfections with its antisense homologue.

1731 retrotransposon; *Drosophila*; Gag protein; Antisense

## 1. INTRODUCTION

Retrotransposons are mobile genetic elements. They provoke mutations. Found in the genome of all eukaryotes examined so far, they ressemble retroviruses (see [1–7] for reviews). 1731 is a *Drosophila melanogaster* retrotransposon moderately repeated in the fly genome and in that of cultured cells as well. It is 4648 bp long and flanked by 336 bp long terminal repeats (LTRs) of U3-R-U5 architecture. The 1731 internal sequence consists of two long open reading frames (ORF). ORF1 and 2 present similitaries with retroviral *gag* and *pol* genes respectively [8,9]. Functional dissections of the LTRs showed a core promoter followed downstream by a silencer-like sequence and preceded upstream by a powerful activator fragment required for the negative regulation by the steroid hormone, 20 hydroxyecdysone [10,11]. The unidirectional transcription generates a major full-length polyadenylated RNA [9–12]. Added to the demonstration that, expressed in *E. coli*, the *pol* gene encodes an authentic reverse transcriptase [13], the existence of 1731 RNA/DNA duplexes [14] and of double stranded DNA extra-chromosomic forms [8] is also reminiscent of vertebrate retroviruses.

As the translation of a 1731 gag protein remained an unsolved problem, we tagged an entire element by fusing its ORF1 with the *E. coli* lacZ gene either in frame (sense polarity) or in antisense polarity. After transfections in *Drosophila melanogaster* (S2), *Drosophila hydei* or *Drosophila virilis* (DV1) cultured cells, it is only the $\beta$-galactosidase activity of the sense construction which was observed. The high transfection efficiency of DV1 cells we observed, allowed the findings that, through immunodetection of its gag moiety, the correctly translated chimeric protein was gathered in cytoplasmic virus-like particles (VLPs) whereas, thanks to the enzymatic amplification, it could be also detected in the nucleus. Extending the antisense methology (e.g. [15–19]) to retrotranposons we found experimental conditions for inhibiting the $\beta$-galactosidase activity of the entire sense construction by cotransfection with its antisense homologue.

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## 2. MATERIALS AND METHODS

### 2.1 *Plasmid constructions*

1731-lacZ recombinant plasmids were prepared as shown (Fig. 1). From the pCH110 plasmid [20] the *Kpn*I–*Bam*HI fragment containing the LacZ gene was inserted into the *Kpn*I–*Bam*HI digested pTrc99C plasmid [21]. From this intermediate construct, the *Nco*I–*Bam*HI was made blunt and inserted into the unique *Nru*I site of pFP6C (= pUC8–1731) plasmid [8]. The resulting construction referred to as pKM8+ is thus an entire 1731 harbouring the first 9/10 of the ORF1 fused in frame with the *E. coli* lacZ gene when inserted in the sense orientation defined by that of the ORF 1. Alternatively, the lac Z gene was inserted in an opposite (antisense) orientation: pKM 8− plasmid.

### 2.2. *Cell lines*

*Drosophila melanogaster* S2 cells [22] and *Drosophila virilis* (DV1) cells [23] were grown in Schneider's medium (Gibco) supplemented with 10% fetal calf serum. *Drosophila hydei* (KUN-DH33) were grown in M3 medium [24]. Southern blotting of DNA extracted from *D. virilis* or *D. hydei* cells showed us the absence of 1731 in their genomes. (not shown)

### 2.3 *Transfection experiments*

Transfection or cotransfection experiments were performed by the

calcium phosphate coprecipitation technique as previously described [10]. The amount of transfected DNA is expressed in μg DNA/flask i.e. per 107 cells.

2.4. *β-Galactosidase assay*

The β-galactosidase activity in cell extracts from transfected cells was measured using orthonitrophenylgalactoside (ONPG) as described [25]. In order to visualize the β-galactosidase activity into the cells, 48h after transfection, slightly fixed cells were stained by the X-gal method [26]. The β-galactosidase positive blue stained cells were numbered through an inverted microscope. At least 3,000 cells were counted to obtain a percentage of blue cells (BC) with an S.D. of ± 2%.

2.5. *Sucrose gradient analysis and Western blots*

In order to follow the fate of the 1731 gag chimeric gene product and to characterize virus-like particles (VLPs), extracts of transfected DV1 cells were analyzed by Western blotting of fractions separated on a sucrose density gradient [13] 1731 gag proteins were recognized by specific anti-gag antibodies we generated in guinea-pigs against the central part of the gag protein produced in *E. coli* from the *Bam*HI–*Pst*I fragment of pFP6C (Fig. 1)

## 3. RESULTS

### 3.1. *The chimeric gag-LacZ gene of entire tagged 1731 is expressed in transfected Drosophila cells*

After pKM8+ transfections in either S2, DV1 or DH33 cells, an increase of β-galactosidase activity, measured with OPNG, was found in the first two cells types. Undetectable in hydei cells, it was 4- to 6-fold higher in virilis cells than in melanogaster ones. With pKM8− and whatever the species of cells transfected, this increase could not be detected. Because the endogenous β-galactosidase activity [27] hindered this technique, we choose the X-Gal method which, although it required tedious numbering of the blue cells, allowed quantification of the transfection efficiency and the cellular localization of the reporter enzyme. The reliability of the method was checked: (1) the endogenous background was undetectable and remained so after pKM8− transfection in the three cells species; (2) the percentage of blue cells (BC) correlated with the enzymatic activity measured with OPNG. For given amounts of pKM8+ transfected the percentage of DV1 BC was at least 5-fold higher than that of S2 and still much higher than that of BC hydei cells (≤ 0.1%). BC microscopic examinations were performed. The staining was heterogeneous, from deep to light blue. It could be scattered between the cytoplasm and the nucleus or confined in only one of these two compartments.

### 3.2. *The percentage of β-galactosidase-producing cells depends on the amount of transfected pKM8+ and the time after transfection*

Using the DV1 cells, we analyzed the blue cells percentage as a function of the amount (μg) of transfected pKM8+ DNA (Fig. 2). The resulting curve has a sigmoidal shape including a plateau reached for 30 mg of pKM8+. This plateau is preceded by a nearly linear variation with a steep slope: the percentage of blue cells is increased 3 times for a 1.5 (30/20) DNA variation. In order to appreciate the stability of the β-galactosidase expression as a function of time after transfection, DV1 cells, transfected once with pKM8+, were sub-cultured each week during two months. The percentage of blue cells decreased in function of the number of dividing cells. Thus, we limited our experiments to the 48 h following transfections, at the moment of the highest BC percentage.

### 3.3. *Characterization of the 1731-gag-β-galactosidase produced in Drosophila cells*

Was the observed β-galactosidase activity produced by the chimeric gag-β-galactosidase we expected to be found in the cells? In order to answer this question we transfected about 109 DV1 cells with pKM8+ DNA. 48 h later, cell extracts were prepared, fractionated on a sucrose gradient and analysed by immunoblotting. Fig. 3 shows the presence of a band detected by anti-gag antibodies, with an apparent MW of 160 kDa, consistent with the predicted MW for the chimeric protein (145 kDa). This indicated that the chimeric gag gene is correctly translated. Moreover, we observed that the chimeric protein was located in gradient fractions (no. 6–9) with densities around 1.22g/ml, characteristic of VLP-containing fractions in *Drosophila melanogaster* cells ([28] and references therein). Another band of MW ~ 200 kDa was also recognized in these fractions. It could represent aggregated forms of the gag-β-galactosidase.

### 3.4. *β-Galactosidase expression in cells cotransfected with plasmids pKM 8+ and pKM 8−*

Fig. 4A shows the effect of pKM8− DNA when cotransfected with a given quantity (30 μg) of pKM8+ DNA. The DV1 percentage of blue cells progressively decreases with increasing amounts of pKM8−: e.g. for ratios sense/ antisense between 3 and 0.5, 6 % of blue cells are detected, i.e. an inhibition of 55%. As control experiments, cells were cotransfected with pKM8+ and pUC8 (the *E. coli* vector of pKM8+). Results (Fig. 4A) indicated that no inhibitory effect could be detected for pUC8 quantity up to 30 μg, (a total amount of 60 μg of DNA). Rather, a slight increase of the blue cells percentage was sometimes observed. A cytotoxicity-like decrease in blue cells percentage occurred only for amounts of cotransfected DNA greater than 60 μg. In an inverse experiment (Fig. 4B, inset) pKM8- quantity was kept constant (30 μg). Increasing amounts of pKM8+ provoked a progressive increase of the percentage of BC. At a ratio of 1/1 the percentage of blue cells was equal to that found in the above experiment: 6% of cells were expressing β-galactosidase.

In the experiments reported in Fig. 5, the total DNA quantity used for the cotransfections was kept constant (30 μg). In contrast with the control upper curve, cotransfected with various pKM8−/pKM8+ DNA ratios (lower curve), the percentage of blue cells rapidly

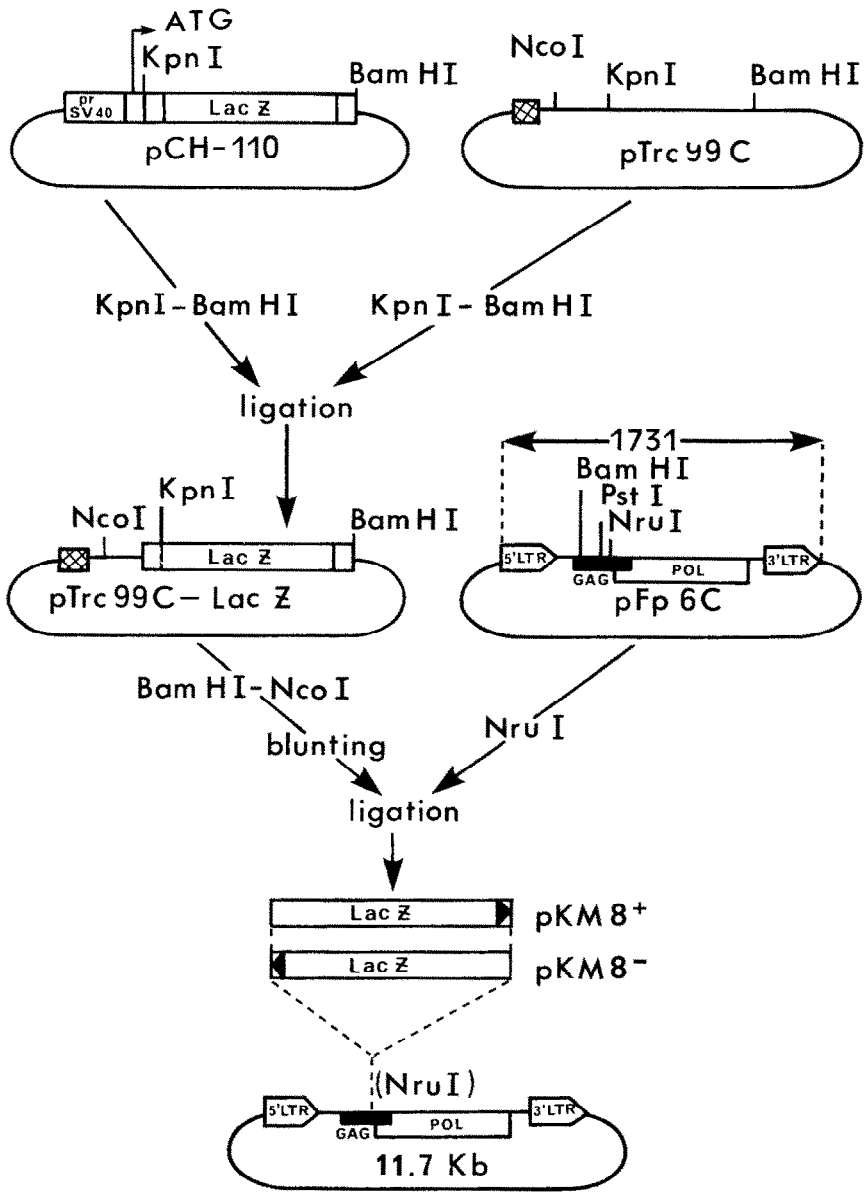


Fig. 1. Construction of plasmids pKM8+ and pKM8- (see Materials and Methods).

decreased when the ratios increased. Inhibition of the β-galactosidase expression was comprised between 65% and 85% for DNA ratios of 0.2 to 5.

## 4. DISCUSSION

Translations of both gag and pol genes of retrotransposons are postulated requirements for their mobility [1–7]. The expression, in *E. coli*, of an enzymatically active reverse transcriptase encoded by the 1731 pol gene was the first demonstration that a retrotransposon of high eukaryotes is able to fulfill at least one of these requirements [13]. Characterization of the translation product of the 1731 gag gene was needed.

Although the genome of *Drosophila melanogaster* cultured cells contains about 30 integrated copies [8] such a study was severely hindered by the rareness of the translation products of 1731. In order to overcome this difficulty, we tagged an entire 1731, modified as little as possible, by fusing in frame an *E. coli* reporter gene with the 9/10 upstream part of its gag gene, hoping to benefit from the enzymatic amplification (pKM8+ plasmid, Fig. 1). After transfection, backgrounds due to the endogenous β-galactosidase activity [27] led us to select the X-Gal staining method the reliability of which was cheked as explained in results. The occurrence of staining, in three different *Drosophila* species cells, demonstrated the translation of the pKM8+ chimeric gene. Comparisons between this efficient sense construction and the silent antisense one (pKM8−) indicate that the

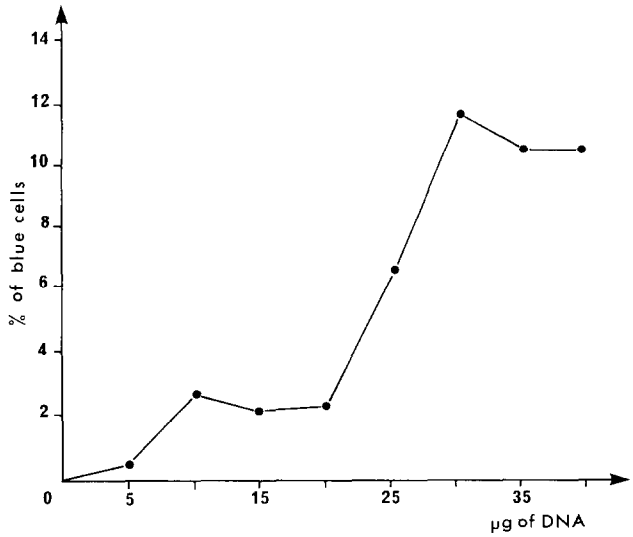


Fig. 2. The percentage of $\beta$-galactosidase-expressing cells is dependent on the quantity of transfected pKM8+. 48 h post-transfection, cells were stained by the X-gal method and counted. The percentages of DV1 blue cells are given as a function of the amount of transfected pKM8+ DNA.

promoter is located into the 5'LTR. This is consistent with the unidirectional transcription of 1731 (see [9] and antisense experiments below) and observations of our laboratory showing that short constructions containing

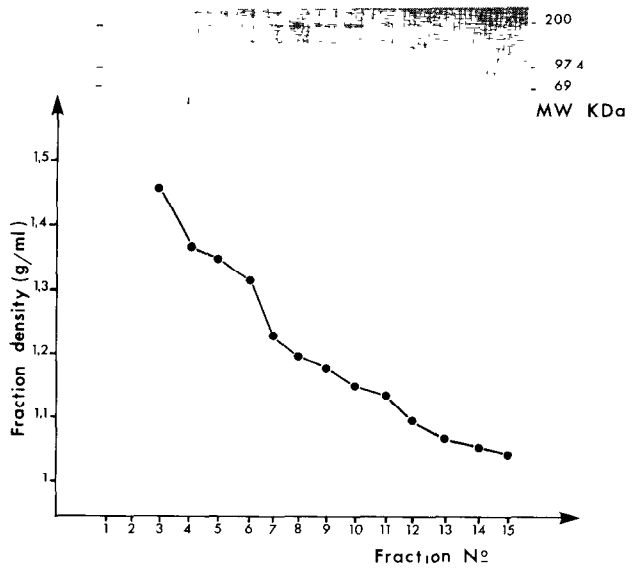


Fig. 3. Presence of 1731 gag-$\beta$-galactosidase chimeric protein in pKM8+ transfected *Drosophila virilis* cells. 30 flasks of DV1 cells were transfected with pKM8+ DNA. 48 h after transfection, the cells were harvested, rinsed and homogenized in Tris buffer containing proteases inhibitors. A 15,000 × $g$ supernatant was deposited over a sucrose density step gradient (20–30–70%). The gradient was fractionated and analysed through electrophoresis and immunoblotting. The upper part of the figure shows the result of an immunoblotting with a specific anti-gag antiserum revealed by DAB staining. The lower part of the figure represents the density profile of the sucrose gradient, expressed in g/ml.

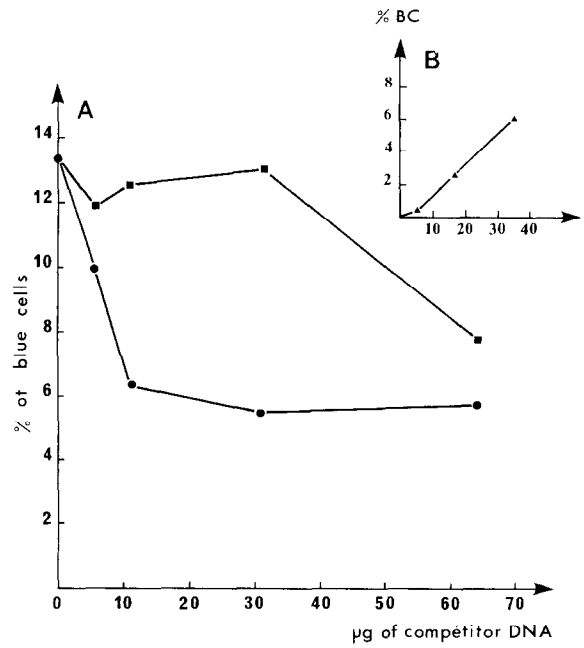


Fig. 4. Expression of sense pKM8+ is inhibited when it is cotransfected with antisense pKM9− For cotransfecting DV1 cells, pKM8+ was maintained constant (30 $\mu$g) and the quantity of pUC8 (■—■) or pKM8− (●—●) varied from 0 to 65 $\mu$g Blue cells were counted and their percentages are given as a function of the quantity of competitor DNA(4A). 4B (inset): the percentages of blue cells (%BC) increase when increasing amounts of pKM8+ are cotransfected with a constant quantity (30 $\mu$g) of pKM8−.

only the entire 5'LTR or its 232 bp internal part (referred to as B9 or L2, respectively [11]) immediately followed by the chloramphenicolacetyltransferase (CAT) gene are sufficient for promoting the CAT activity even in DV1 cells. In this respect, the characterization, in both S2 and DV1 cells, of NssBF (Nuclear single strand Binding Factor) which specifically interacts with a fragment of the mRNA-like strand of the U3 region of the 1731 core promoter [29] appears meaningful.

It has also to be noted that 1731 DNA double stranded, extrachromosomic full-length forms, harbouring LTRs, have been characterized [8]. They might be translated. Promoted by the LTR, the transient expression of an entire transfected retrotransposon provides a model strengthening such an hypothesis difficult, if not impossible, to demonstrate otherwise.

Finally, from an evolutionary point of view, the persistence, however variable, of the 1731 promoter function in cells of species which do not contain this retrotransposon bring experimental data in favour of hypothetical horizontal transfers, i.e. transmission of retrovirus-(like) elements from a species to another one (e.g. [5,6,30,31]).

The chimeric gag gene of pKM8+ is about 5-fold longer than the natural one. The DV1 cells properties (high transfection efficiency and absence of 1731) al-

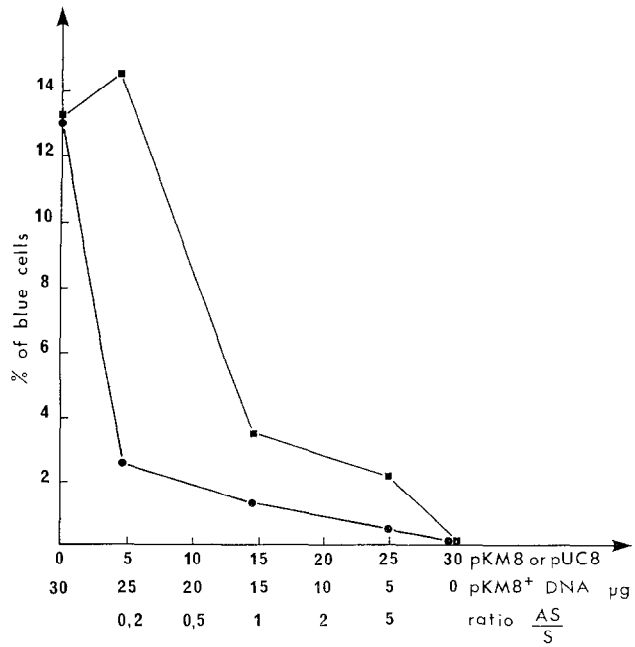


Fig 5. Inhibition of the pKM8+ expression as a function of ratio between antisense and sense constructions The total amount of cotransfected DNA in DV1 cells is kept constant (30 $\mu$g). It is the sum of pKM8+ and pUC 8 or that of pKM8+ and pKM8−. The blue cell percentages are depicted as a function of the ratios between the quantities ($\mu$g) of the corresponding cotransfected DNA i.e. pUC8/pKM8+ (■—■) or pKM8−/pKM8+ (●—●).

lowed the demonstration of its correct translation by using antigag antibodies. The fused gag protein is gathered in cytoplasmic (Fig. 3) VLPs containing fractions. Even modified the 1731 gag protein could be one of the keystones in building these VLPs. Its homologies with HIV [9] suggest some similar mechanisms [32] involving at least the 1731 gag nucleic acid binding site (1731 NBS : V V C Y N C G E R R H F K A N C; homologies with HIV NBS underlined) located just upstream the *Nru*I site and a putative packaging region of the RNA leader sequence separated by 23 (21 in HIV) ribonucleotides from the AUG begining the gag ORF (1731 : A G A U U U U G G ...23...AUG; homologies with HIV underlined)

Thanks to the enzymatic amplification the presence of the 1731 chimeric gag protein can be evokated in the nucleus of several transfected cells. Consistent with this histological localization is the finding of a nuclear localization signal (NLS : A K K R K D shared by e.g. SV40 large T, frog Nuclear protein N1 or human Lamin A; for review see [33]) located (position signal: 218–223) upstream the gag NBS. Current efforts are made in our laboratory for demonstrating if this NLS is required/sufficient for the transport of the 1731 gag protein across the nuclear envelope.

Retrotransposons set specific problems. Each of their numerous and normally integrated copies is liable to be transcribed and translated. They are mobile but their roles during cellular differentiation and development are subjects of controversy. For the study of such populations taken as ensembles and to have a chance for interacting with all the members of a given family it seemed us that interfering with their RNAs could be one of the most convenient way. We thus checked if the antisense techniques [15–19] could be adapted to retrotransposons by cotransfecting the entire 1731 tagged by the sense lacZ gene with its antisense homologue.

During the restatement of the method, first results (Fig. 4) showed that, for better characterizing antisense effects, DNA overload has to be avoided. On the other hand, the linear part of the reference curve (Fig. 2) seems to exclude a limiting availability of the transcription machinery. Moreover, its steep slope defines the best conditions for dealing with antisenses. The value of 30 $\mu$g was thus chosen as total amount of transfected DNA and kept constant. A clear cut, between control points and experimental ones, has then been obtained (Fig. 5).

Altogether the data draw a more complete picture of 1731. The transposition, in *Drosophila melanogaster* cells [34], of this retrovirus-like element can be relinked to the enzymatic properties of its reverse transcriptase [13] and now to translations and fates of its gag protein. Here applied in cultured cells to 1731, the antisense techniques should provide a helpful tool of general interest, e.g. in transgenic flies.

*Acknowledgements*: Thanks are due to C. Montmory and D. Rouillé for expert technical assistance. This work was supported by the Centre National de la Recherche Scientifique (CNRS) and by grants to M.B.B. from l'Association pour la Recherche sur le Cancer (ARC), from the Ligue Natonale Française contre le Cancer, from the Institut National pour la Santé et la Recherche Médicale (INSERM), and from the Fondation pour la Recherche Medicale (FRM).

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