Nucleic Acids Research, 1993, Vol. 21, No. 14 **3265–3268**

Two *Drosophila* retrotransposon *gypsy* subfamilies differ in ability to produce new DNA copies via reverse transcription in *Drosophila* cultured cells

Natalia V.Lyubomirskaya, Sergei N.Avedisov, Sergei A.Surkov and Yurii V.Ilyin
V.A.Engelghardt Institute of Molecular Biology, Academy of Sciences of Russia, Vavilov str. 32, Moscow 117984, Russia

Received March 3, 1993; Revised and Accepted June 7, 1993

ABSTRACT

Plasmid DNA constructs containing 5'end truncated retrotransposon *gypsy* were introduced into *Drosophila* cultured cells. Appearance of new complete DNA copies with reconstructed via reverse transcription 5'LTR were detected by PCR after transient expression and by Southern blot analysis of genome DNA of stably transformed cells. Two *gypsy* subfamilies supposed to be different in transpositional activity were analyzed in terms of their ability to produce new DNA copies via reverse transcription in *D.hydei* cultured cells. It was demonstrated that both *gypsy* variants undergo retrotransposition but with different efficiency.

INTRODUCTION

Retrotransposons are a widely distributed group of eukaryotic mobile genetic elements that resemble the vertebrate retroviruses in their structure and mode of replication. *Gypsy* (mdg4) (1, 2) is one of the most thoroughly studied *Drosophila melanogaster* retrotransposons transpositional cycle of which is based on reverse transcription (3). Recently it was shown that *gypsy* plays an important role in the genetic system of Mutator Strain that exhibits prolonged instability (4, 5). Molecular analysis of the structural organization of *gypsy* elements cloned from both Mutator Strain (MS) and Stable Strain (SS), from which MS originated, revealed the existence of two distinct subfamilies, which have definite structural differences (6). These differences are mainly single nucleotide substitutions and most of those in coding region do not change aminoacids sequence. In total there are only 16 aminoacids substitutions. The data obtained showed that in spite the fact that both *gypsy* variants are transcriptionally active only one of them is amplified in *Drosophila melanogaster* cultured cells (1) and in MS (6) and causes insertional mutations (2, 7–10). This allowed us to suggest that these two *gypsy* subfamilies differ in transpositional activity and ability to produce new DNA copies via reverse transcription. To check this suggestion we have created a model system allowing us to detect the appearance of new *gypsy* elements formed via reverse transcription and compared the ability of two *gypsy* variants to produce new copies in *Drosophila* cultured cells. The latter were detected by Southern blot analysis in stably transformed cells and by PCR after transient expression.

MATERIALS AND METHODS

Preparation and treatment of nucleic acids

Extraction of plasmid, total genomic DNA and poly(A)$^+$RNA, restriction enzyme treatment, DNA labelling, Southern and Northern blotting experiments were performed according to Maniatis et al. (11).

DNA constructs

The initial construct pHS*GYP* described elsewhere (12) contained 5'end truncated *gypsy* under control of the hsp70 promoter. p*GYP*(Xho)6 was constructed by inserting 6.6 kb *XhoI–EcoRI* fragment and 0.8 kb *EcoRI–HindIII* fragment from pHS*GYP* into pUC19 treated with *SalGI* and *HindIII*. As a result p*GYP*(Xho) contained only one *XhoI* site in the *gypsy* 3'LTR. p*GYP*(Xho)7 was constructed by ligating the 6.7 kb *BglII–XhoI* fragment from p7K (6) with the 3.4 kb *BglII–XhoI* fragment from p*GYP*(Xho)6. p*GYP*(p) was constructed in several steps. Two *gypsy* fragments: 0.2 kb *XhoI–XbaI* and 0.6 *XbaI–Sau3A* from Dm111 (1) were inserted into pUC19 treated with *SalGI* and *BamHI*. This intermediate I construct was treated with *SmaI* and *EcoRI* and ligated with 0.25 kb *EcoRI–BspRI* fragment containing complete polylinker from pUC19. The next step was the insertion of two *gypsy* fragments: 1.3 kb *Sau3A–PstI* and 4.3 kb *PstI–EcoRI* into the *BamHI* and *EcoRI* sites of intermediate II construct. And finally the 2.2 kb *EcoRI* fragment from Dm111 was inserted in the proper orientation into the *EcoRI* site of intermediate III construct.

Transfection

D.melanogaster Schneider 2 and *D.hydei* Dh14 (13) cultured cells were transfected by a standard calcium phosphate procedure (14) by plasmid DNA of analyzed constructs in the presence of

pUChsneo (15) DNA. Stably transformed cells were obtained as being resistant to G418.

PCR

Primer oligonucleotides were synthesized using an applied Biosystems 381A synthesizer. Transfected *D.hydei* cells (10^6) were collected by centrifugation, washed with PBS, lysed in 100 μl of PCR non-ionic buffer (50mM KCl; 10mM tris−HCl pH 8.3; 2.5mM $MgCl_2$; 0.1 mg/ml gelatin; 0.45% NP-40; 0.45% Tween20) containing proteinase K (60 μg/ml) and incubated at 37°C for 1 hour and at 90°C for 10 min. 10 μl of cell extracts were used for PCR reactions. 20 cycles (denaturation at 95°C, 1 min, annealing at 45°C, 1 min, polymerization at 72°C, 1 min) were performed in PCR non-ionic buffer containing 250 μM dNTP, 0.25 μM primers and 2.5 units of *Taq* polymerase (USB). Reaction products were separated in 1.5% agarose gels and visualized under UV illumination.

RESULTS

The structure of plasmid constructs

Fig.1 shows the schematic presentation of plasmid constructs used. They contain *gypsy* with the 5'LTR truncated up to the position of −30, relative to the RNA start site (3). Thus, such constructs do not contain a complete *gypsy* element but they can be transcribed, producing normal, complete *gypsy* RNA. Hence after the cycle of reverse transcription in cultured cells there should appear full-length *gypsy* DNA containing reconstructed 5'LTRs, which can be detected directly by PCR and by the appearance of *gypsy* DNA containing *XhoI* sites in both LTRs in Southern blot experiments using DNA from stably transformed cells.

p*GYP*(Xho)6 construct was made for *D.hydei* Dh14 line cells and contained *gypsy* under the control of the *D.melanogaster* hsp70 promoter. p*GYP*(p) was made for *D.melanogaster* Schneider2 line cells. To distinguish the introduced construct from endogenous copies the former contained *gypsy* marked by a 250 bp pUC19 fragment inserted into a *Sau3A* site located between the regulatory region and ORF1 ATG codon of the element.

Introduced plasmid constructs produce new *gypsy* DNA copies

p*GYP*(Xho)6 and p*GYP*(p) constructs were introduced into *D.hydei* Dh14 and *D.melanogaster* Schneider2 cultured cells respectively. In both cases transfection was performed in the presence of pUChsneo DNA (15). Stably transformed cells were obtained as resistant to G418. DNA isolated from transformed cells was digested by *XhoI* and analyzed by Southern blot technique. The results of these experiments are presented in Fig.2. Two main bands can be seen with both Dh14 (Fig.2a, lane 2) cells after the hybridization to *gypsy* DNA and Schneider2 cells (Fig.2b, lane 7) after the hybridization to 250 bp fragment of pUC19 DNA, while DNA isolated from untransformed cells did not hybridize to probes used (Fig.2a, lane 5 and Fig.2b, lane 6). The upper 10.1 kb (Fig.2a, lane 2), which has the same size as initial plasmid construct treated with *XhoI* (Fig.2a, lane 3), and 11.55 kb (Fig.2b, lane 7) correspond the introduced constructs inserted as tandem repeats. The lower 7.0 kb (Fig.2a, lane 2), which has the same size as wild-type *gypsy* treated with *XhoI* (Fig.2a, lane 1) and 7.25 kb (Fig.2b, lane 7) bands correspond newly formed *gypsy* copies. Thus, these experiments showed that introduced *gypsy* constructs are able to produce new

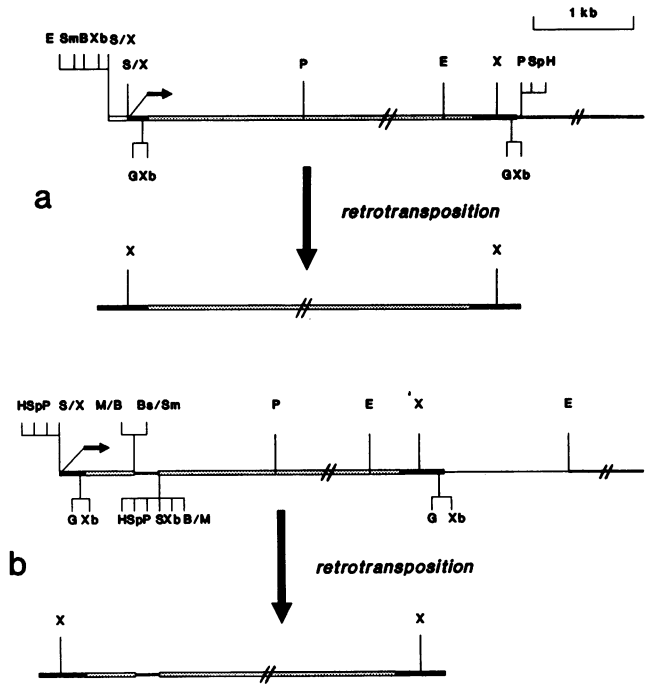

Figure 1. Schematic presentation of DNA constructs used in research. LTRs are denoted by a solid line, hsp70 promoter sequences by an open line, *gypsy* internal sequences by a dotted line, adjacent *D.melanogaster* sequence by a thin line and pUC19 by a thick line. RNA start site is indicated by arrow. Restriction enzymes are abbrivated as follows: B, *BamHI*; Bs, *BspRI*; E, *EcoRI*; G, *BglII*; H, *HindIII*; M, *Sau3A*; P, *PstI*; S, *SalGI*; Sm, *SmaI*; Sp, *SphI*; X, *XhoI*; Xb, *XbaI*.

DNA copies containing 5'LTR reconstructed via reverse transcription both in *D.hydei* and *D.melanogaster* cultured cells.

Only *D.hydei* cells known to lack normal *gypsy* element were used in further experiments to exclude the possibility of interaction between introduced and endogenous *gypsy* copies.

Both *gypsy* variants undergo reverse transcription but with different efficiency

To compare ability of two *gypsy* variants to produce new DNA copies via reverse transcription we have used two constructs: p*GYP*(Xho)6 based on Dm111 similar to p6K, cloned from MS and p*GYP*(Xho)7 based on p7K, isolated from SS (6). First of all we intended to check if p*GYP*(Xho)7 also can undergo reverse transcription in *D.hydei* cells. For this purpose we have performed PCR reactions on DNA isolated from *D.hydei* cells 48 hours after being transfected either by p*GYP*(Xho)6 or p*GYP*(Xho)7. As first primer the oligonucleotide from 5'end of LTR (position 115) was used. The introduced constructs contain this sequence only in 3'LTR. As the other primer the oligonucleotide adjacent to 5'LTR (position 519) was used. The results of these experiments (data not shown) revealed the appearance of DNA fragments of the expected size in *D.hydei* cells transfected by both p*GYP*(Xho)6 and p*GYP*(Xho)7 while in untransfected cells such fragments were absent. Thus, it was demonstrated that both *gypsy* variants undergo reverse transcription in *D.hydei* cells. Still these experiments could not answer the question if newly formed copies can be integrated into genome DNA and if there is any difference in the efficiency of the processes. To answer these questions we obtained *D.hydei* cells stably transformed by p*GYP*(Xho)6 and p*GYP*(Xho)7.

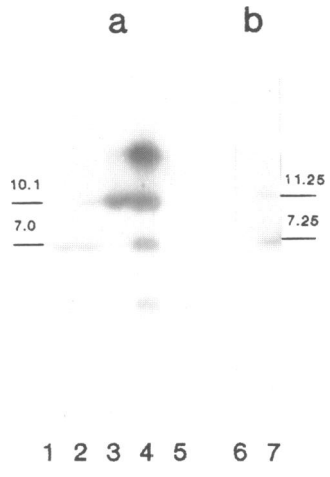

Figure 2. Southern blot analysis of *XhoI* digested DNA of plasmid p*GYP*(Xho)6 (lane 3) and DNA isolated from different cell lines: *D.melanogaster* Schneider2 (lanes 1, 6); *D.hydei* (lane 5); *D.hydei* transformed by p*GYP*(Xho)6 (lane 2) and *D.melanogaster* transformed by p*GYP*(p) (lane 7). As a probe ^{32}P-labelled *XhoI gypsy* fragment (**a**) and 250 bp *BspRI-EcoRI* pUC19 fragment (**b**) were used. ^{32}P-end-labelled lamda *HindIII* fragments (lane 4) used as size markers are: 23.1 kb; 9.4 kb; 6.7 kb and 4.4 kb.

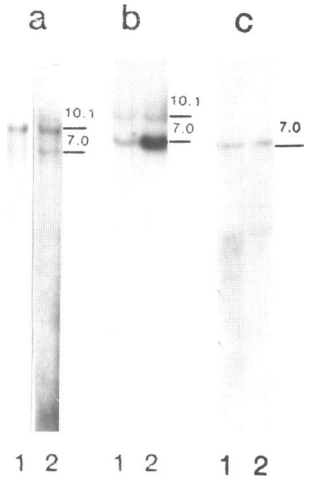

Figure 3. Hybridization of ^{32}P-labelled *XhoI gypsy* fragment to *XhoI* digested DNA (**a** and **b**) and poly(A)$^+$RNA (**c**) isolated from *D.hydei* cells transfected by p*GYP*(Xho)7 (1) and p*GYP*(Xho)6 (2). Nucleic acids were isolated immediately as cell lines were obtained (a and c) and 6 weeks later (b).

Southern blot analysis of DNA isolated from cell lines Dh*GYP*(Xho)6 and Dh*GYP*(Xho)7 revealed that both *gypsy* variants can produce new DNA copies integrated into genome DNA but with different efficiency. The results of such experiments slightly varied from one to another. Results of Southern blot analysis of DNA obtained in one of the most impressive experiments, as well as Northern blot analysis of poly(A)$^+$RNA isolated from these cell lines, are presented in Fig.3. Sometimes the appearance of new *gypsy* copies in Dh*GYP*(Xho)7 was detected immediately after the cell lines were obtained. Sometimes new copies were detected only several weeks later. In the case of Dh*GYP*(Xho)6 newly formed *gypsy* copies were always detected immediately after the cell lines were obtained. Moreover, the ratio between the intensity of the 7.0 kb *XhoI* fragment corresponding to newly formed *gypsy* copies and 10.1 kb *XhoI* fragment corresponding to the introduced constructs was always less in the case of Dh*GYP*(Xho)7 cell lines than for Dh*GYP*(Xho)6 lines (Fig.3a). Therefore, both *gypsy* variants undergo reverse transcription in *D.hydei* cells and produce new DNA copies but in case of p*GYP*(Xho)6 this process is several times more efficient than in case of p*GYP*(Xho)7.

Southern blot analysis of the same cell lines 6 weeks later (Fig.3b) demonstrated that in both cell lines *gypsy* is amplified and again in Dh*GYP*(Xho)6 this process is more efficient than in Dh*GYP*(Xho)7.

It can be seen that there is practically no difference in transcription of introduced *gypsy* constructs in both cell lines (Fig.3c) in spite the fact that there may be some variations in copy number of the element. Therefore, the difference in ability to produce new DNA copies via reverse transcription between two *gypsy* variants cannot be explained by difference in their transcription.

DISCUSSION

The present paper is devoted to comparative analysis of two *gypsy* subfamilies in terms of their ability to produce new DNA copies. We have created a model system which allowed us to detect *gypsy* DNA originated via reverse transcription of its RNA. Analogous systems were made for Ty1 (16) and I elements (17, 18). It is noteworthy that we have shown the appearance of *gypsy* DNA containing restored 5'LTR using neither selective markers nor the conditions of hyperexpression. For *D.melanogaster* cells we used constructs containing only *gypsy* promoter elements adjacent to RNA start site. The only reason why we used hsp70 promoter in constructs for *D.hydei* cells was the possibility that *gypsy* promoter would not work in heterologous system. No heat shock induction was used. Thus, the processes of *gypsy* reverse transcription in *Drosophila* cultured cells are rather efficient.

The comparison of two *gypsy* variants in these experiments revealed that both are able to undergo reverse transcription but with different efficiency. Variations in experiments may be due to influence of nonspecific reverse transcriptase or some uncontrolled conditions of cell transformation. To exclude the latter we have obtained *D.hydei* cultured cells line containing both *gypsy* constructs. Southern blot analysis of DNA isolated from such cells confirmed the results described above. To check the possibility of interaction between introduced constructs and some endogenous *D.hydei* reverse transcriptases we made a construct based on p*GYP*(Xho)6 with interrupted ORF2. The preliminary results of our experiments showed that reverse transcription of such construct can be detected only by PCR but not by Southern blot analysis.

Data obtained do not exclude the possibility that in flies only one *gypsy* subfamily (which was cloned from MS) is 'active' and can transpose. Sequence analysis (data will be published elsewhere) of p7K, cloned from SS (6) showed that 'inactive' *gypsy* has identical LTRs indicating that this element either was 'active' recently or still can undergo reverse transcription with low efficiency. This *gypsy* variant has several structural differences in comparison to p6K (6). The latter is similar to Dm111 (1) and *gypsy* cloned from *forked* mutation (2). As it was mentioned above there are only 16 aminoacids substitutions in all three ORFs. All the nucleotide substitutions in noncoding regions do not touch important regulatory areas and as it was

demonstrated previously (6) and in this work do not effect *gypsy* transcription. Therefore, we consider that some (or maybe one) of aminoacids substitutions are responsible for the difference in transpositional activity. Some of these 16 substitutions seem to be of no importance changing one aminoacid to another of the same type (e.g. Val–Ile). Two substitutions are located between the first and the second ATG codons of ORF1 and it seems that the latter is a real translation start site. There are two substitutions of Pro, one in the end of ORF1 and the other in the end of ORF2, which may change the structure of protein products of these genes. Among the most interesting substitutions are the following. The first one is located in the beginning of ORF3 (there are 6 substitutions in this ORF in total) which changes Gln to termination codon. But it is located between two theoretical acceptor splice sites, so if *gypsy* ORF3 is expressed like retroviral *env* gene trough splicing using the first one acceptor splice site, then this substitution may be significant. The second interesting substitution is located in the overlap region of ORF1 and ORF2 and may influence the frameshift and as a result the ratio between *gag* and *gag-pol* protein products.

We believe that usage of hybrid constructs, containing different combinations of 'active' and 'inactive' *gypsy* copies in mentioned above model system will allow us to find particular structural differences responsible for the lower transpositional activity of 'inactive' *gypsy*.

ACKNOWLEDGEMENTS

We are grateful to Dr Andrew Flavell for help and fruitful discussion and to Alastair Murchie for oligonucleotide synthesis. This research was supported by Russian State Program 'Frontiers in Genetics'. Some of experiments were performed in Department of Biochemistry, Dundee. N.L. was supported by EMBO Postdoctoral Fellowship.

REFERENCES

1. Bayev, A.A., Lyubomirskaya, N.V., Dzhumagaliev, E.B., Ananiev, E.V., Amiantova, I.G., Ilyin, Y.V. (1985) *Nucl. Acids Res.* **12**, 3707–3723.
2. Marlor, R.L., Parkhurst, S.M., Corces, V.G. (1986) *Mol. Cell. Biol.* **6**, 1129–1134.
3. Arkhipova, I.R., Mazo, A.M., Cherkasova, V.A., Gorelova, T.V., Shuppe, N.G., Ilyin, Y.V. (1986) *Cell* **44**, 555–563.
4. Kim, A.I., Belyaeva, E.S., Aslanyan, M.M. (1990) *Mol. Gen. Genet.* **224**, 303–308.
5. Kim, A.I. and Belyaeva, E.S. (1991) *Mol. Gen. Genet.* **229**, 437–444.
6. Lyubomirskaya, N.V., Arkhipova, I.R., Ilyin, Y.V., Kim, A.I. (1990) *Mol. Gen. Genet.* **223**, 305–309.
7. Modolell, J., Bender, W., Meselson, M.M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1678–1682.
8. Mizrokhi, L.J., Obolenkova, L.A., Priimagi, A.F., Ilyin, Y.V., Gerasimova, T.I., Georgiev, G.P. (1985) *EMBO J.* **4**, 3781–3787.
9. Peifer, M. and Bender, W. (1986) *EMBO J.* **5**, 2293–2303.
10. Peifer, M. and Bender, W. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9650–9654.
11. Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
12. Lyubomirskaya, N.V., Arkhipova, I.R., Ilyin, Y.V. (1989) *Genetika (Russia)* **26**, 1720–1728.
13. Sondermajer, P. (1980) *In vitro* **16**, 913–919.
14. DiNocera, P.P. and Dawid, I.B. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7095–7099.
15. Steller, H. and Pirotta, V. (1985) *EMBO J.* **4**, 167–171.
16. Boeke, J.D., Garfinkel, D.J., Styles, C.A., Fink, G.R. (1985) *Cell* **40**, 791–500.
17. Pellison, A., Finnegan, D.J., Bucheton, A. (1991) *Proc. Natl. Acad. Sci. USA.* **88**, 4907–4910.
18. Jensen, S. and Heidmann, T. (1991) *EMBO J.* **10**, 1927-1937.