ORIGINAL ARTICLES

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Chromatin organisation of transgenes in *Dictyostelium*

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Dedicated to Prof. Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.

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The introduction of transgenes in *Dictyostelium discoideum* typically results in the integration of the transformation vector into the genome at one or a few insertion sites as tandem arrays of approximately 100 copies. Exceptions are extrachromosomal vectors, which do not integrate into chromosomes, and vectors containing resistance markers such as blasticidin, which integrate as single copies at one or a few sites. Here we report that low copy number vector inserts display typical euchromatic features while high copy number insertions are enriched for modifications associate with heterochromatin. Interestingly, high copy number insertions also colocalise with heterochromatin, are enriched for the centromeric histone CenH3 and display centromere-like behaviour during mitosis. We also found that the chromatin organisation on extrachromosmal transgenes is different from those integrated into the chromosomes.

1. Introduction

Since the development of a functional transformation system in *Dictyostelium* (Nellen et al. 1984) a multitude of vectors has been constructed that lead to various levels of gene expression. For integrating vectors it appears that the copy number of the integrated transgene largely determines its expression level (Nellen and Firtel 1985; Egelhoff et al. 1989). Copy number in turn appears to depend on the efficiency of the resistance cassette. While neomycin and hygromycin (Egelhoff et al. 1989) resistance appear to be weak and require many (approx. 100) copies to confer resistance to the selective agent, blasicidine resistance (Sutoh 1993) is strong and only one or a few copies are needed to confer resistance. Extrachromosomal vectors based on the Ddp1 extrachromosomal maintenance region (Firtel et al. 1985) typically occur at approx. 100 copies per cell, independent of the resistance cassette.

The correlation between copy number and expression is nonlinear and high copy insertions of G418 resistance plasmids are typically expressed at levels 10 to 100 fold lower than one would expect from approx. 100 transgene copies driven by the actin 6 or actin 15 promoter.

One possible explanation for this observation is that the tandem copies of the inserted vector may trigger transgene silencing as has been previously reported in plants (Assaad et al. 1993; Aufsatz et al. 2002).

To further investigate this possibility we examined the chromatin organisation of transgenes in *Dictyostelium discoideum.* Immunofluorescence microscopy and ChIP assays with antibodies against a typical euchromatic ($H3K4^{me2}$) and a typical heterochromatic ($H3K9^{me3}$) histone modification were employed in combination with fluorescence in situ hybridisation (FISH).

2. Investigations and results

2.1. Integrating high copy plasmids

We first used a cell line transformed with the vector pGEM-G418 that has approximately 100 integrated copies (Nellen and Firtel 1985 and data not shown). FISH with a probe against the transgene and against the centromeric DIRS-1 retrotransposon was performed to investigate their relative localization.

In prophase the transgene probe labels four foci (Fig. 1A, bottom) suggesting at least four independent transgene integration sites. However during interphase, the transgene appears at a single focus mostly colocalised with the centromere probe (Fig. 1A, top).

This pattern is reminiscent of the centromeric DIRS-1 retrotransposon, which although present at the centromeres of each of the five chromosomes is clustered in a single focus adjacent to the centrosome during interphase. It should be noted, however, that the overlap between the DIRS-1 and transgene probes is only partial. Similar localisation patterns were observed in over 10 independent multi-copy transgenic cell lines containing between one to five insertion sites (see Table 1).

The distribution of histone modifications on the transgenes was examined using chromatin immunoprecipitaion (ChIP).

In a cell line containing the pDneo2a-GFP fusion construct, a copy number of approx. 100 was determined by absolute quantification with real time PCR (data not shown). As expected for an expressed gene, ChIP with an antibody against the euchromatin marker $H3K4me^2$ followed by PCR on the GFP transgene showed a signal (Fig. 1C). However, even stronger enrichment of the GFP transgene was detected in chromatin precipitated with antibodies against the heterochromatin marker $H3K9me^3$ (Fig. 1B). Control PCR's against the DIRS-1 retrotransposon gave signals similar to those previously reported (Dubin et al.

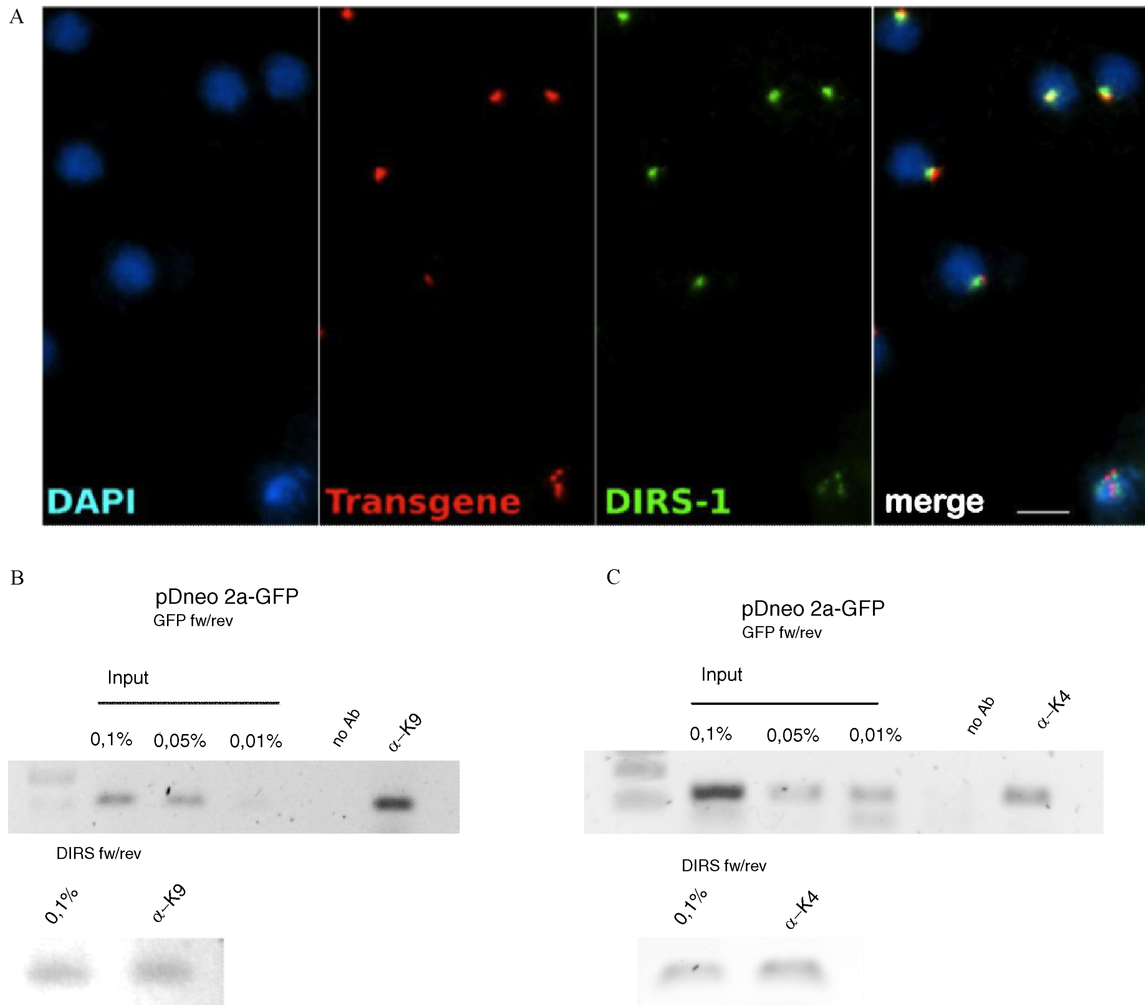


Fig. 1: High copy plasmids co-localise to heterochromatin. **A**: Fluorescence in situ hybridisation (FISH) on cells containing integrated high copy number transgenes. Directly FITC-labelled probes for DIRS-1 and Cy3-labelled probes for the transgene were used. During interphase the transgenes co-localises at least partially to heterochromatic regions (see overlay DIRS-1/transgene) while with the beginning of prophase, the transgene(s) are visible as discrete foci along one or more chromosomes (see cell at the bottom) Scale Bar = 5 μM **B:** ChIP with an antibody against the heterochromatic marker H3K9me[3]. SemiquantitavePCR (27 Cycles) was performed against the Myc-tag of the integrated transgene (GFP). **C:**ChIP with an antibody against the euchromatic marker H3K4me[2]. Only a weak PCR signal for the transgene is obtained. No Ab = no antibody.Control PCRs were done with primers against DIRS-1-ORF2

2010). Independently transformed cell lines resulted in similar data (not shown). Thus, high copy transgenes are, to a large proportion, heterochromatic.
The largely heterochromatic character of high copy transgenes could be due to the transgenes preferentially inserting into heterchromatic regions (which are largely confined to the centromeres and telomeres in *Dictyostelium*). Alternatively, silencing factors could be recruited for *de novo* silencing of the transgenes. To distinguish between these possibilities we carefully examined the localisation of a transgene, in this case a single insertion through the mitotic cycle.
During mitosis the six centromeres separate into distinct spots that are stained by a DIRS-1 probe. The transgene, in this case a single site integration, is seen as a discrete focus approximately 1 μm apart from the centromere (Fig. 2), indicating that it has not integrated into the centromeric region but rather loops back to the cluster of heterochromatic centromeres during interphase. As cells enter metaphase, the chromosomes become more

Table 1: Additional transgenic lines used in this study

Line	Vector	Copy Number	Numbers of Insertions	Localisation
pG418-5	pGEM-G418	100	4	pericentromeric
pG48-8	pGEM-G418	100	?	pericentromeric
pLTR1	pGEM-LTR	10	?	euchromatin
agoAas15	pDneo2-agoAas	approx 100	1	pericentromeric
pGEM-E1	pGEM-G418	approx 100	1	pericentromeric
pGEM-E2	pGEM-G418	approx 100	2	pericentromeric
pGEM-E4	pGEM-G418	approx 100	1	pericentromeric
pGEM-E5	pGEM-G418	approx 100	1	pericentromeric
pGEM-F1	pGEM-G418	approx 100	2	pericentromeric
pGEM-F2	pGEM-G418	approx 100	2	pericentromeric
pGEM-F3	pGEM-G418	approx 100	4	pericentromeric
pGEM-F4	pGEM-G418	approx 100	3	pericentromeric

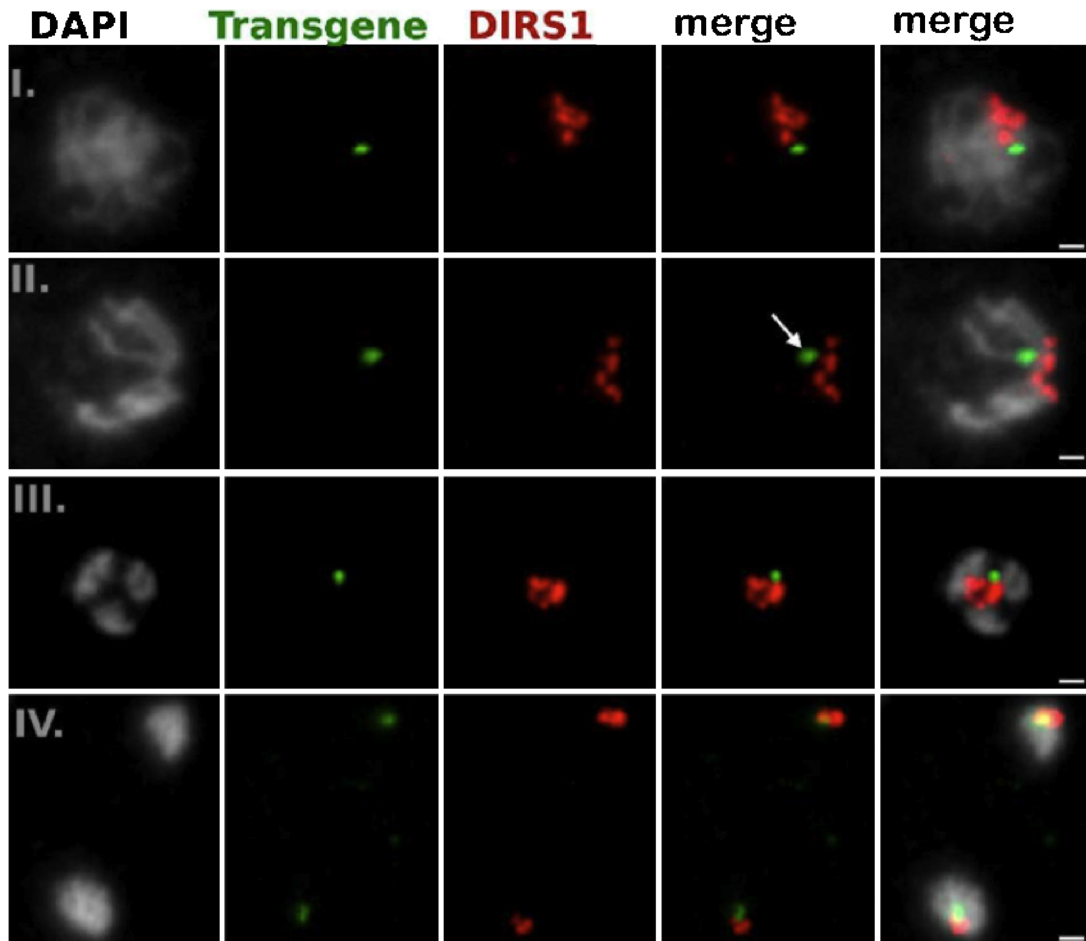


Fig. 2: High copy transgene localisation during the cell cycle.FISH on interphase cells where a transgene of high copy number (approximately 100) has integrated at a single locus. A Cy3-labelled probe for DIRS-1 and an FITC-labelled probe for the transgene were used. Different stages of the cell cycle demonstrate the behaviour of the transgene during mitosis. I: early prophase, II: prophase, III: metaphase, IV: telophase. The arrow indicates the position of the transgene on a prophase chromosome Scale bar = 1 μm

compact and centromeres organize in a ring like structure around the microtubule spindle. The transgene locus appears to cluster in this ring together with the other centromeres. During telophase the transgene locus trails slightly behind the leading centromeres.

Notably, high copy transgenes integrated at multiple sites display a single focus in interphase but, similar to the centromeres split up in mitosis (see Fig. 1 A). The similarity of high copy transgenes with centromeres in respect to localization and heterochromatisation prompted us to examine a potential association with the centromeric histone H3 (CenH3). As seen in Fig. 3, Cy3 labelled CenH3 (Dubin et al. 2010) partially overlaps with transgene staining by FISH. ChIP analysis shows that the transgenes are clearly co-precipitated by an antibody against the tagged CenH3.

2.2. *Integrating low copy transgenes*

To see if heterochromatin formation occurred on all transgenes or if it was a function of copy number we examined cells transformed with an integrating pDbsr2a-Myc construct conferring blasticidin resistance. The copy number was firstly determined by qPCR (data not shown) and found to be approx. 1 copy per cell. In contrast to high copy transgenes, low copy insertions (1 up to 10 copies per cell) were found in euchromatic territories by double FISH experiments comparing DIRS-1 and transgene localization (Fig. 4A). In immunoprecipitates with an antibody against a euchromatic modification (H3K4me^2), the transgene was clearly detected (Fig. 4C) while almost no signal was seen in precipitates by an anti H3K9me^3 antibody (Fig. 3B).

2.3. *Extrachromosomal transgenes*

An alternative strategy for expressing transgenes at high levels in *Dictyostelium* is the use of plasmids containing the Ddp1 extrachromosomal maintenance region. These extrachromosomal constructs display about 80–100 copies per nucleus (Firtel et al. 1985). They have high transformation efficiency and expression levels that are usually somewhat higher than from high copy integrating vectors but still below those expected from the copy number.

FISH experiments showed a dispersed distribution of the extrachromosomal transgenes all over the nucleus (data not shown). This may reflect the true distribution of the extrachromsomal transgenes but is more likely due to a disruption of a plasmid cluster by the FISH preparation (see discussion).

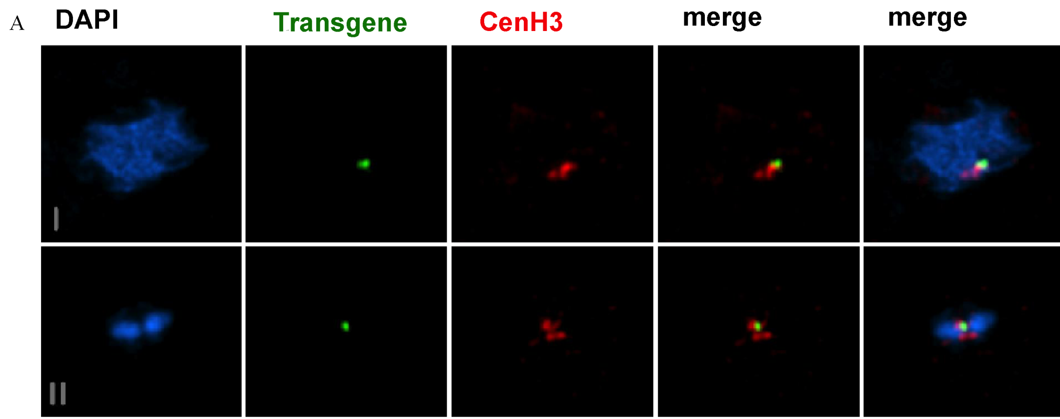
To confirm the presence of nucleosomes on the extrachromsomal transgenes we performed ChIP on a cell line transformed with both the extrachromsomal pDbsrXP-GFP construct and an integrating histone H2B-Myc fusion on a pDNeo2a vector. Using primers against the GFP fragment of the extrachromsomal vector we showed that there is at least some association of H2B with the extrachromosomal constructs (Fig. 5C).

However in contrast to the integrating constructs we were unable to detect either H3K9me^3 or H3K4me^2 on the extrachromosomal transgenes (Fig. 5A and B).

3. Discussion

The level of expression of a transgene can be influenced by many parameters such as the site of genome integration and chromatin

A



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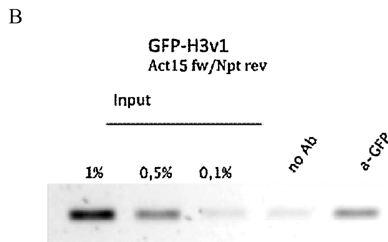
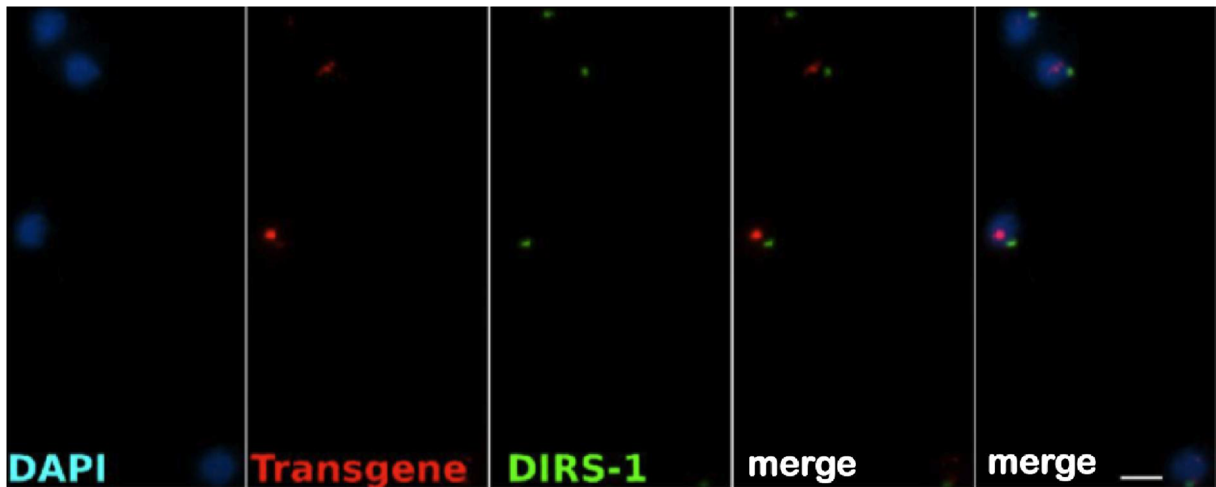


Fig. 3: High copy transgenes appear as centromere-like structures. **A:** FISH on cells containing a CenH3-GFP high copy transgene. A Cy3 labelled probe against CenH3 and a FITC labelled probe for the transgene were used. I: early prophase, II metaphase. **B:** ChIP analysis with an antibody against GFP. SemiquantitativePCR (25 Cycles) was performed with primers inthe promotor and the resistance cassette of the transgene construct. No Ab = no antibody



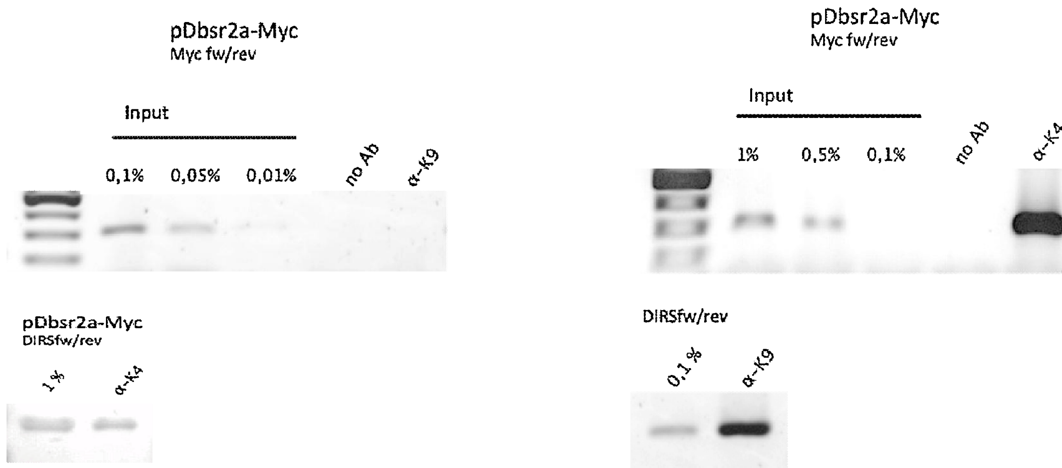


Fig. 4: Euchromatic association of low copy plasmids. **A:** FISH on interphase cells with a low copy plasmid integrated into the genome. An FITC-labelled probe was used for DIRS-1 and a Cy3-labelled probe to detect the transgene. Scale Bar = 5 µM. **B:**ChIP with an antibody against the heterochromatic mark H3K9me[3]. **C:**ChIP with an antibody against the euchromatic mark H3K4me[2]. For both assays, semiquantitave PCR (28 Cycles) was performed for the tag of the integrated transgene (6xMyc). Control PCRs were done with primers against DIRS-1-ORF1. No Ab = no antibody

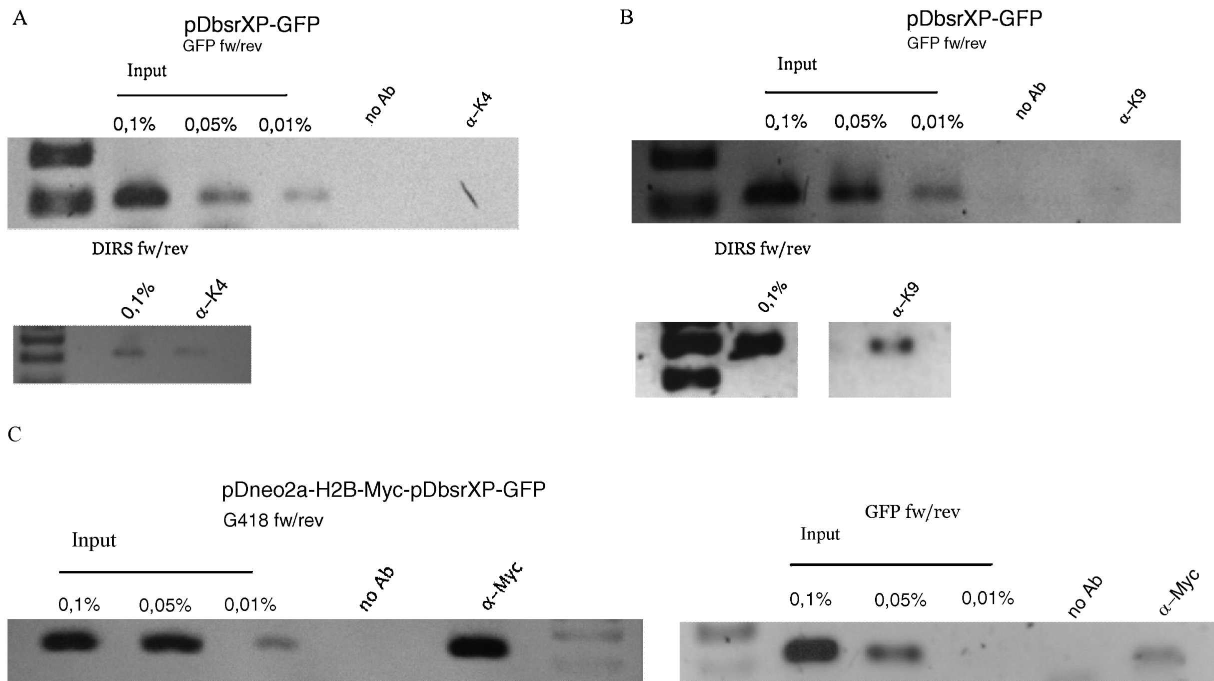


Fig. 5: Organisation of extrachromosomal plasmids. **A**: ChIP with an antibody against H3K4me^2. **B:** ChIP with an antibody against H3K9me^3. Semiquantitave PCRs (27 Cycles) were performed against the tag of the extrachromosomal plasmid (GFP). Control PCRs were done with Primers against DIRS-1. **C:** ChIP on a cell line containing an H2B-Myc plasmid and an extrachromosomal GFP containing plasmid. Immunoprecipitation was done with a Myc Antibody. PCRs were than performed with primers for the H2B-Myc plasmid (G418 resistance primers) and for GFP from the extrachromosomal plasmid. While the extrachromsomal plasmid is clearly associated with H2B, no significant signal was obtained from immunoprecipitates with antibodies against the H3 modifications

organisation. In *Dictyostelium* high copy number transgenes are normally expressed at levels far below the expected values given their copy number. Several mechanisms could account for the low expression levels observed. Transgenes may generate siRNAs that eventually result in DNA methylation and chromatin remodelling (repeat induced silencing, Assaad et al. 1993; Pelisson et al. 2007). We have investigated siRNA generation and could not detect any transgene derived small regulatory RNAs. Similarly we have analysed DNA methylation by bisulfite sequencing and did not find any significant DNA methylation at these sites (data not shown).

With antibodies directed against specific chromatin marks we have shown that multi-copy insertion vectors have modifications associated with heterochromatin and that the transgene localise close to the centromeres during interphase. However from examining prophase chromosomes it is clear that the transgenes are not inserted into centromeric repeats. Rather it appears that the transgene array triggers de novo heterochromatin at the site of insertion and may thus induce looping back to associate with centromeric regions during interphase.

In metaphase the transgene heterochromatin appeared to arrange in the ring like structure together with the centromeres. Immunofluorescence and ChIP analysis showed that transgenes were at least partially associated with the centromeric histone variant CenH3 suggesting that they constitute centromere-like structures. Since we did not observe significant perturbations in mitosis and there are no reports in the literature that transgenic strains in general have a growth disadvantage, we conclude that the presence of CenH3 is not sufficient to functionally specify a centromere and that the transgene centromere-like structures do not significantly disturb the distribution of chromatids in mitosis.

In *Dictyostelium* there are no reports on heterochromatin spreading. It can, however, not be ruled out that transgenes may influence gene expression in the vicinity of their integration site by position effect variegation. It would be of interest to investigate the chromatin organisation and expression of chromosomal genes adjacent to the transgene integration site.

It has been assumed that high copy integration of plasmids carrying a neomycin resistance was required to compensate for a weak phosphotransferase activity of the protein. Since tandem integrations apparently induce heterochromatin, our data suggest that a subtle balance has to be maintained since more copies may actually result in more gene silencing. Obviously, only a portion of the transgenes are euchromatic and active but we do not know if these copies are interspersed in the array, clustered or located in the borders. A similar situation is observed with the chromosomal DIRS-1 copies that are clustered in the centromeres (Dubin et al. 2010; Glockner et al. 2001) and which are in part heterochromatic and in part euchromatic.

While many species have genomes consisting largely of heterochromatin (animals, many plant species such as maize, tomato) others such as *Dictyostelium* and *Arabidopsis* have very little heterochromatin which is largely confined to the centromeres. This suggests that by some mechanism repetitive elements (e.g. transposons) are preferentially eliminated. Here we show that repetitive transgene arrays become heterochromatised, are enriched in the centromeric histone CenH3 and form centromere-like structures.

In many cases increasing the selection pressure can enhance expression levels of transgenes but the underlying molecular mechanisms are unclear. One may speculate that for some reason heterochromatic marks are reduced and more genes in the array become active.

Low copy transgenes strongly contrast the high copy ones: no heterochromatic histone marks are detected and we have never seen an association of the transgene with centromeric heterochromatin in interphase cells.

FISH experiments on extrachromosomal transgenes showed a dispersed staining all over the nucleus, similar to staining the rDNA which is arranged in extrachromosomal linear palindromes. We believe that this is an artefact of the FISH preparation since at least the rDNA palindromes are found clustered in a chromosome like body in cells that have been prepared

by other means. Probably, extrachromosomal plasmids are also arranged in a cluster that is disrupted by the preparation.
Plasmids are frequently found to have non-canonical chromatin. In *Dictyostelium* they appear to lack the most common chromatin marks: we could not detect $H3K9me^3$ or $H3K4me^2$. Only for histone H2B we could show that extrachromosomal plasmids are organised as chromatin. Chubb and colleagues have shown that developmental gene expression correlates very well with specific epigenetic chromatin marks (Chubb et al. 2006). Since these are not detectable on extrachromosomal plasmids, this implies that other regulatory mechanisms like promoter specificity are sufficient to control the expression of plasmid born genes. Though we have not further analysed chromatin composition of extrachromosomal plasmids, the data demonstrate that epigenetic regulation differs from that at chromosomal loci.

4. Experimental

4.1. Vectors and primer

Transgenes that were used for Fish analysis: high copy pGEM-G418, low copy pGEM-LTR, pDneo2a-GFP-H3v1
Transgenes used for ChIP analyses are described in Dubin and Nellen (2010) (pDneo2a GFP, pDbsr2a6xMyc, pDbsrXPGFP)
All PCR recations were done with Taq Polymerase using the following primers:
qGFPfor: GAAAACTACCTGTTCCATGGC (SM57_gfp_qPCR_for)
qGFPrev: GGCATGGCACTCTTGAAAAAG (SM58_gfp_qPCR_rev)
6xMycfor: CAGGTCGACGGTATGGATCCAAAGCTATGGAGC (MJD9)
6xMycrev: GTACCGGATCCCGTCGACCCGGAATTCAGGTCC (MJD-14)
DIRS1for: GGAAGAAGAAAGCCCCATTC (MJD83)
DIRS1rev: CAGAGAAGCCATAGCGGAAC (MJD84)
G418for: GGTTTAAAAAAACTTGGGTTGG (CHAct15)
G418rev: CATTATCGCGAGCCCATTTA (MJD61)
Act15for: GGTTTAAAAAAACTTGGGTTGG (CHAct15)
NptIrev: AGAGCTTTGTTGTAGGTGGA (MJD42)

4.2. *Dictyostelium* cells and culture

The *D. discoideum* strain Ax2-214 (axeA2, axeB2, axeC2, Ochiai et al. 1982) was cultured in HL5 (ForMedium) containing 50 μg/ml, 0,25 μg/ml of amphotericin-B, 100 μg/ml Penstrep (PAA) and the appropriate selective agent (10 μg/ml of geneticin and/or 10 μg/ml of blasticidin). Alternatively, *Dictyostelium* cells were grown on bacterial lawns of *Klebsiella aerogenes* on SM agar plates. *Dictyostelium* Ax2 cells were transformed using electroporation (Howard et al. 1988) and selected in $HL5^+$ containing the appropriate antibiotics. Suspension cultures were incubated on a rotary shaker (150 rpm) to a densitiy of 2×10^6 cells/ml.

4.3. Copy number determination

Copy numbers were determined with absolute quantification using real time PCR. Whole cell DNA from the respective transformants was isolated from $2x10^8$ *Dictyostelium discoideum* cells. Briefly, cells were pelleted by centrifugation, resuspended in 45 ml icecold lysisbuffer (50 mM Hepes pH 7,5, 40 mM $MgCl_2$, 20 mM KCl, 5% sucrose), NP40 was added drop wise with shaking until cells were lysed (up to 1%). Nuclei were spun down by centrifugation for 15 min at 5000 rcf. Samples were resuspended in 5 ml lysis buffer without NP40 and digested with proteinase K (20 mg/ml) for 3 h at 60 °C. The lysate was extracted with phenol/chloroform and the aqueous phase was precipitated with cold ethanol. The precipitated DNA was washed with 70% ethanol, dried and resuspended in 100 μl H_2O.
As reference for the absolute quantification exact determination of the respective plasmid and copy number per μl were done. Absolute quantification of copy number was done using the SensiFast SybrGreen Kit from Bioline on an Eppendorf realplex light cycler. Copy number calculation was done according to the formula (Lee et al. 2005):

$$\text{DNA copy} = \frac{6.02 \times 1023\,(\text{copy/mo}) \times \text{DNA amount}\,(\text{g})}{\text{DNA length}\,(\text{dp}) \times 660(\text{g/mol/dp})}$$

Fluorescence *in situ* hybridisation (FISH) was done as previously described by Dubin et al. (2010).

4.4. Chromatin immunoprecipitation (ChIP)

ChIP was done according to Dubin et al., 2010 except that the following buffers were used: ChIP Lysis buffer: 20 mM Tris/HCl pH 7, 150 mM NaCl, 0.5% NP40, 3 mM $MgCl_2$, proteinase inhibitoren complete Mini Roche (1 tablet per 10 ml lysis buffer), wash buffer: 100 mM TrisHCl pH 7, 150 mM KCL, 2 mM $MgCl_2$ RIPA wash buffer: 50 mM Tris/Cl, pH 7.5, 150 mM NaCl, 0.5 % NP40, 0.5% Sodium Deoxycholate, 1 mM EDTA, 0.1% SDS Dilution buffer: 20 mM Tris/HCl pH 7, 150 mM NaCl, 3 mM $MgCl_2$ The following antibodies were used: anti-$H3K4me^2$ (Epitomics #1347-1), anti $H3K9me^3$ (Millipore #07-523), anti Myc (9E10) PCR reactions were performed in 50 μl reactions containing Taq polymerase, 1.5 mM $MgCl_2$, 2 mM dNTPs, 5 pmol of each primer and 1 μl of sample DNA from each immunoprecipitation. For detection of GFP, G418 and DIRS-1 following program was used: 94 °C 3 min once; 94 °C 30 s, 60 °C 1 min for multiple cycles and then once at 60 °C 3 min. For detection of Myc following programm was used: 94 °C 3 min once; 94 °C 30 s, 53 °C 1 min, 72 °C 1 min, for multiple cycles and then once at 72 °C 3 min.
The cycle number for each PCR was determined by multiple runs choosing a program where the elution fraction gave a clear signal while the "no antibody" control did not. PCR products were analysed on ethidium bromide stained 1% agarose/TBE gel.

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