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Modification of the methylation pattern in the vicinity of the chicken globin genes in avian erythroblastosis virus transformed cells

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ABSTRACT

Having previously found a reduced transcription of globin genes and an abortive processing of the already transcribed globin pre-mRNA in Avian Erythroblastosis Virus (AEV) transformed cells (1), we compared the genomic DNA of these cells with that of normal chicken erythroblasts, using ^{32}-P-labelled cDNA probes specific for the β, α^A and α^D globin sequences. Restriction endonuclease digestion, electrophoresis of digests in agarose gels, Southern blotting and hybridization were carried out.

Our results show that the overall genome organization is not disturbed in the immediate neighbourhood of the adult globin genes ; the observed restriction fragments are identical for both DNAs after EcoRI, HindIII, BamHI and XbaI digestion, using the β, α^A and α^D globin cDNA probes.

However, we observe specific modifications at some methylation sites in the β, β-like and α^D regions : after HpaII or MspI digestion in the α^D region and after HhaI digestion in the β and β-like region, heavier bands appear in the transformed cell DNA in addition to the ones observed in normal DNA. This implies that, at some specific sites, the transformed cell DNA is more methylated than the normal erythroblast DNA. The possible significance of this observation is discussed.

INTRODUCTION

In Avian Erythroblastosis Virus (AEV) transformed cells maintained in culture, synthesis of hemoglobin is undetectable and no trace of globin mRNA appears in the cytoplasm. However, globin genes are transcribed into nuclear pre-mRNA (1). Recent results indicate that only α^A globin transcripts are detectable (2). In searching for a possible explanation of the abortive processing of globin specific pre-mRNA, we were prompted to investigate the globin transcriptional unit at the DNA level. Profound perturbation of chromosome organization is known to occur in cells transformed by leukemia virus (3,4). Thus, we had to ascertain whether the globin gene organization is altered or not in the transformed cells. Therefore, we compared,by restriction enzyme digestion,DNA from chicken hemo-

poïetic cells transformed by AEV, cultured for ten days in vitro, with DNA from normal chicken erythroblasts. The genomic fragments were separated by electrophoresis on agarose gels, transferred onto nitrocellulose sheets (5) and hybridized with ^{32}P labelled probes, i.e. chicken β, α^A and α^D globin inserts of pBR322 recombinant plasmids (6).

In addition to the study of the existence of a possible disturbance of the DNA organization by the viral genome in the immediate neighbourhood of the globin genes, we also analyzed the level of DNA methylation at some points of the regions surrounding and including the β, β-like, α^A and α^D globin genes by the use of restriction enzymes sensitive to the methylation of their cleavage site (7).

From the recent work of Engel and Dodgson it is known that α^A and α^D chicken globin genes are separated by 2.4 kb in the genome (8). As regards the β genes, it has been shown that the adult β gene is linked to three β-like embryonic genes not yet fully characterized, the whole cluster spanning about 15 kb (9,10 and Dolan, Dodgson and Engel, unpublished results cited in 11).

Variations in the methylation pattern within and around globin genes in different tissues have been reported in chicken (12,13), in man (7) and in rabbit (14). In transformed cells, the methylation of proviral genomes (15,16) and the level of methylation of the DNA of the transformed tissue itself (17) has been investigated. However, these studies have not yet been extended to a specific cellular gene whose function is affected after transformation by an RNA tumor virus. We show here that although the overall genome organization is undisturbed in the vicinity of the globin genes, their degree of methylation at some specific sites is increased in the AEV transformed cells.

MATERIALS AND METHODS

Cells : In-bred leukosis free brown Leghorn chicken were used. Induction of erythroleukemia with AEV and the in vitro culture of the transformed erythroblasts are described elsewhere (1). Normal erythroblasts were taken from anemic chickens, after three daily injections of acetylphenylhydrazine (15 mg/ml in NaCl 0.9% - 1 ml/kg).

Preparation of DNA : High molecular weight DNA was prepared as described elsewhere (18).

Enzymatic digestion of DNA : Restriction enzymes were supplied by Bethesda Research Laboratories, New England Biolabs, Boehringer-Mannheim and by M.

Meier (I.R.B.M., Paris). DNA was digested according to the producers instructions. To ensure the completeness of the digestion, we tested increasing concentrations of enzyme and times of digestion (e.g. for 5 µg DNA samples from 20 units enzyme, 2 hours up to 60 units, 5 hours at 37°C).

Agarose gel electrophoresis : We used 0.6% and 0.9% horizontal agarose gels (19). Each sample contained 5 µg of hydrolyzed DNA. EcoRI and HindIII digested phage λ DNA were used as markers. After electrophoresis, the gels were stained with 2 µg/ml ethidium bromide in the electrophoresis buffer and photographed under short wavelength U.V. illumination.

DNA transfer by Southern blotting : DNA was denatured *in situ* in 1.5 M NaCl, 0.5 M NaOH. The pH was lowered to 10.7 by washing with 3 M NaCl and the DNA was transferred in 3 M NaCl (pH 10.7) (20) overnight at room temperature according to the Southern procedure (5). Nitrocellulose sheets (Schleicher and Schull, BA 85) were washed in 2xSSC, then dried in vacuum for 2 hours at 80°C (1xSSC is 0.15 M NaCl, 15 mM tri-sodium citrate).

Hybridization : Pre-hybridization and hybridization were performed according to Wahl *et al.* (21). Chicken β, α^A and α^D globin sequences excised from pBR 322 recombinant plasmids (6) were ^{32}P labelled by nick-translation as described (22) and used to probe the restriction enzyme digested DNA. Specific activity obtained was of the order of 10^8cpm/µg.

After hybridization, nitrocellulose sheets were washed three times in 2xSSC, 0.1% SDS at room temperature for 5 min. and two times in 0.1xSSC, 0.1% SDS at 55°C for 20 min. (21).

Autoradiography was performed at -70°C on Fuji-X-Ray films, in presence of Dupont-Cronex "Lightning Plus" intensifying screens.

RESULTS

A summary of our results is given in Table I. Normal erythroblast DNA was prepared in separate experiments from a mixture of anemic blood of ten individual chicken. Transformed cell DNA was prepared in two independent experiments from cells cultured from the pooled blood of four chickens ; all animals were taken from the same breeding stock.

1) Methylation insensitive restriction enzymes : EcoRI, HindIII, BamHI, XbaI.

We did not observe any difference in the number or the intensities of bands of normal and transformed cell DNAs after hybridization with β, α^A and α^D globin specific probes. Our results are in good accordance with those already described for the β globin genes after digestion by EcoRI, HindIII and BamHI (9-11,23-25). For the α^A, α^Dglobin genes, our results confirm the

TABLE I

PROBES	EcoRI		Hind III		Bam HI		Xba I		Hpa II		Msp I		Hha I		Hha I + EcoRI	
	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T
β	9.6 6.7	9.6 6.7	9.6 7 5	9.6 7 5	11 8.5 4.5 2	11 8.5 4.5 2	25	25	4.2 (2.7) 1.3	4.2 (2.7) 1.3	(4) (3.5) 2.7 (2) 1.3 0.65	(4) (3.5) 2.7 (2) 1.3 0.65	7. 4.5 1.3 0.65	7. 5.5 4.5 2.5 1.9 1.3 0.65	6. 3.8 (3.1) 1.3 0.65	6. 3.8 (3.1) 2.5 1.9 1.3 0.65
α^A	12	12	7.6	7.6	2	2	25	25	0.35	0.35	0.35	0.35	1.2 0.7 0.5	1.2 0.7 0.5		
α^D	4.6	4.6	7.6	7.6	1.5 0.8	1.5 0.8	25	25	1.4	1.7 1.4	1.4	1.7 1.4	8.6 7.	8.6 7.	4.6	4.6

Table I : Restriction fragments (in kb) observed after enzymatic digestion of normal chicken erythroblast DNA (N) and Avian Erythroblastosis Virus transformed cell DNA (T) and hybridization with ^{32}P-labelled β, α^A and α^D chicken globin specific probes, i.e. globin inserts of recombinant pBR322 plasmids (6). Main bands are underlined. Faint ones in brackets.

respective localization of α^A and α^D globin genes as proposed by Dodgson et al. (9,8). With stringent washing conditions, we do not observe any cross-hybridization between α^A and α^D probes, nor do we detect other α-like genes, such as the "stress" globin α^A-like gene described (26-28).

The β and α genes clusters are respectively 15 kb and 8 kb long (8-11) in the chicken genome. From the results listed in Table I, and particularly from the XbaI digestions which give very long fragments (25 kb), we conclude that in the transformed cell 1) the AEV genome is certainly not integrated in the close proximity of the β, β-like or α^A-α^D globin gene regions, and, 2) that the globin gene organization at the DNA level is not grossly altered in any other way that might explain the abnormal transcription of these genes and the abortive processing of some transcripts.

2) Methylation sensitive restriction enzymes.

α^A globin gene : The HhaI digestion gives three bands detected by the α^A specific probe in normal and transformed DNA (fig.1). This was predictable from the α^A globin sequence restriction map (6) in the absence of restriction site in the intervening sequences. HpaII and MspI cuts the α^A globin

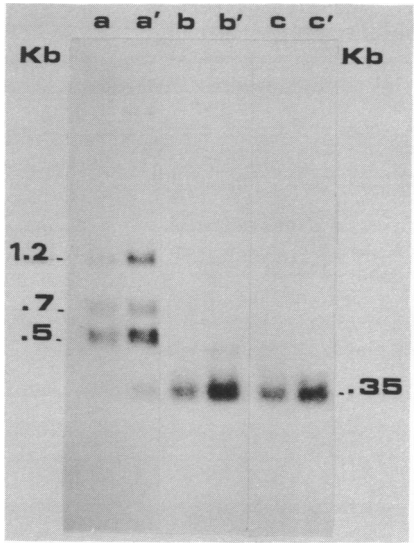

Figure 1 : HhaI digestion pattern of chicken erythroblast DNA (a) and of AEV transformed cell DNA (a') after hybridization with the α^A globin probe. HpaII digestion pattern of normal (b) and transformed DNA (b'), MspI digestion pattern of normal (c) and transformed DNA (c') after hybridization with the α^A globin probe.
5 µg DNA samples were digested for 5 hours at 37°C with 40 units enzyme. Electrophoresis was carried out in a 1% agarose gel and the excised α^A globin sequence of a recombinant cDNA plasmid was used as an hybridization probe.

sequence three times (6), yielding fragments too short to be easily analyzed by the techniques used here. We observe one major 0.35 kb band in all cases (fig.1). Methylation, on the other hand, would produce larger fragments more readily detectable. We conclude that no reproducible and significant differences between normal and transformed cell DNAs, with respect to the α^A globin gene region, are detectable by the use of these three enzymes.

α^D globin gene : HhaI does not cut the α^D globin sequence (6). However, we observe in both normal and transformed cell DNAs two identical fragments hybridizable with the α^D specific probe (8.6 and 7 kb). The reason is not an additional HhaI site in the intervening sequences because the double digestion HhaI-EcoRI gives only the EcoRI fragment (Table I), but it could be due either to a partial methylation of one of the HhaI sites or to an allelic variation.

Significant differences were always obtained in HpaII and MspI digestions : two bands, 1.4 and 1.7 kb long, were consistently observed in the transformed cell DNA. Only the shorter one is observed in normal DNA (fig.2). We know that HpaII cannot cleave the C^mCGG sequence and that MspI cannot cleave the ^mCCGG sequence (7). Our results may be explained if we suppose that, in transformed cell DNA, there is a mixture of $^mC^mCGG$, ^mCCGG, C^mCGG and CCGG sequences at one end of the 1.4 kb fragment. In normal DNA, the HpaII and MspI cleavage site is likely to be unmethylated.

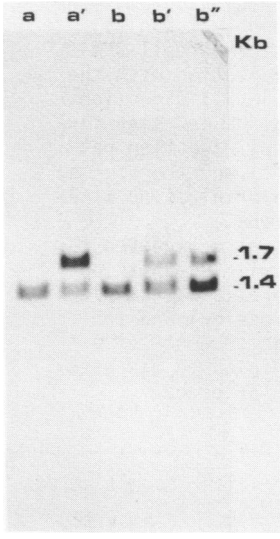

Figure 2 : HpaII digestion pattern of chicken erythroblast DNA (a) and of AEV transformed cell DNA (a') after hybridization with the α^D globin probe. MspI digestion pattern, after hybridization with the same probe, of chicken erythroblast DNA (b) and of two different preparations of AEV transformed cell DNA (b',b"). 5 µg DNA samples were digested with HpaII or MspI (40 units) for 4 hours at 37°C. Electrophoresis was carried in a 0.9% agarose gel and the excised α^D globin sequence of a recombinant cDNA plasmid was used as an hybridization probe.

β and β-like globin genes : HpaII and MspI digests of DNA from normal and transformed cells appeared to be rather similar following hybridization with the β globin specific probe (fig.3). Our results concerning normal DNA are in agreement with those published for chicken erythrocyte DNA (12). The most intense 1.3 kb band may be assigned to the inner part of the β globin gene including the intervening sequences (6). As outlined before (12),

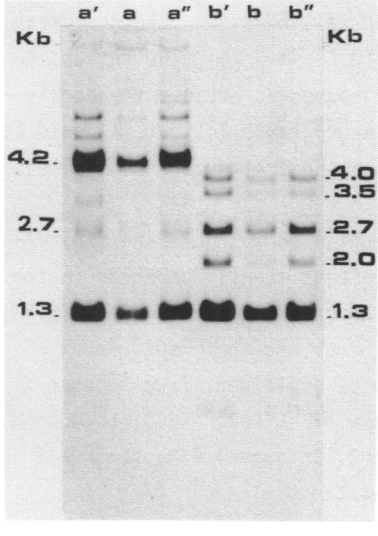

Figure 3 : HpaII digestion pattern of chicken erythroblast DNA (a) and of two different preparations of AEV transformed cell DNA (a',a"), MspI digestion pattern of normal DNA (b) and of transformed cell DNA (b',b") after hybridization with the β globin probe. 5 µg DNA samples were digested for 3 hours at 37°C with 40 units enzyme. Electrophoresis was carried in a 0.9% agarose gel and the excised β globin sequence of a recombinant cDNA plasmid was used as an hybridization probe.

the fact that the HpaII and MspI patterns are different demonstrates the existence of methylation sites around the β globin genes in normal erythroblasts, though the extremities of the 1.3 kb fragment, present in both digests, are not methylated.

With transformed cell DNA, we observe on the contrary reproducible modifications in the pattern of HhaI digestion products as shown in Table I : three additional bands (5.5, 2.5 and 1.9 kb long) were always found (fig.4). As discussed before for the MspI pattern of the α^D region, this implies a higher methylation level of the transformed cell DNA. We compared furthermore the HhaI-EcoRI double digested normal and transformed DNAs : again, the additional 2.5 and 1.9 kb transformed specific fragments were observed (fig.4). The HhaI-EcoRI pattern we observed in normal cell DNA corresponds to that published by Mandel and Chambon (13) for chicken erythrocyte DNA, with, however, some variation in band intensity. These minor variations cannot be attributed to the presence of immature erythroblasts in our cell preparation since we obtain identical patterns for erythrocyte, erythroblast and bone marrow cell DNAs (results not shown).

DISCUSSION

In AEV transformed cells, globin gene transcripts are present in the nuclei, but no globin mRNA appears in the cytoplasm. Furthermore, it was shown that only one of the two main adult globin genes is transcribed (1).

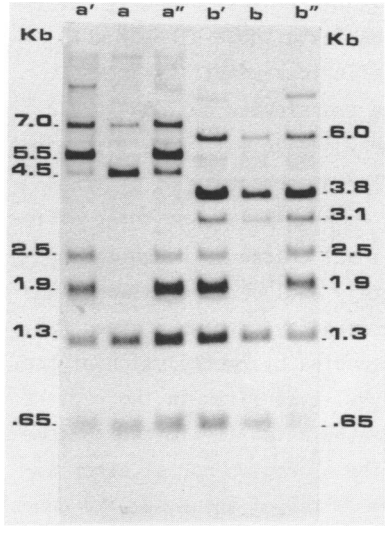

Figure 4 : HhaI digestion pattern of chicken erythroblast DNA (a) and of two different preparations of AEV transformed cell DNA (a', a"), HhaI + EcoRI double digestion pattern of normal DNA (b) and of transformed cell DNA (b', b") after hybridization with the β globin probe. 5 µg DNA samples were digested with HhaI (60 units) or HhaI + EcoRI (50 + 60 units) for 5 hours at 37°C. Electrophoresis and hybridization were performed as described in fig.3.

Recent experiments indicate that the α^A gene is transcribed and the β is not (2). It seems therefore that there are two distinct phenomena : absence of transcription of α^D and β globin genes, and the abortive processing of the already transcribed α^A globin pre-mRNA.

On the basis of our results, we conclude that the AEV genome is neither integrated within the β or the α^A - α^D globin gene regions nor in their close vicinity. Furthermore, the viral transformation has not grossly perturbed in any other way the globin genes and their proximal framing DNA. Since the real length of the globin transcriptional units is still a matter of discussion (29), this conclusion bears evidently only on the presently known globin gene structure. Nevertheless, it is likely that transcriptional and post-transcriptional deviations in globin gene expression cannot be explained merely on the basis of gross gene organization.

Interestingly, we observed in the AEV transformed cell DNA, after digestion with restriction enzymes sensitive to methylation, additional heavier bands which can be explained by a higher level of methylation of specific sites in the neighbourhood of the β, β-like and α^D globin genes. However, two other possible explanations must be considered prior to any definitive conclusion based on our results.

The observed differences could be explained 1) by incomplete digestion of transformed cell DNA : we exclude this possibility having employed exactly the same experimental conditions of digestion for normal and transformed cell DNAs, by increasing enzyme concentrations and digestion times without any variation in our results. Furthermore, different preparations gave the same results and our results are comparable to those already published concerning normal erythrocyte DNA. 2) by allelic variability as was shown for the ovalbumin gene (30) : indeed, the transformed cell DNA could represent a heterozygote, and the normal a homozygote animal. Such a possibility is, however, unlikely, as we used two different preparations of transformed DNA from cells obtained from a total of four animals, and three different preparations of normal DNA from ten individual chicken. Though the possibility of allelic variation cannot be completely excluded, it is worth noting that other workers did not find such heterogeneity of globin genes (23, 31). The observed differences indicate thus a genuine overmethylation of the DNA from transformed cells at some specific methylation sites in the regions including the β and α^D globin genes. This overmethylation is limited to some points as we did not observe modifications in the α^A region nor at other sites of the β and α^D regions, e.g. HpaII cleavage sites of the β, β-like genes

and HhaI cleavage sites of the α^D genes. Furthermore, in the present work, with normal and AEV transformed cell DNA, we never observed differences in the general pattern of DNA digested by methylation sensitive enzymes and analyzed by electrophoresis and ethidium bromide coloration (results not shown), such as the general hypomethylation observed by Craig Cohen in a mouse mammary tumor virus transformed tissue (17).

Many authors agree that genes that are transcriptionnaly active are undermethylated in their DNA, while untranscribed genes are overmethylated (7,13-16,25,32,33). We may also make this correlation, i.e. the overmethylated globin genes are those that are not transcribed in the transformed cell (2).

Two possible hypotheses can be envisaged to explain the modified pattern of methylation of the transformed cells at gene specific loci :
1) We may assume that the transformation event results in the switching-off of otherwise active cellular genes, such as the β adult globin gene and by mechanisms as yet unknown in the modification of the methylation pattern of the target cell DNA. Interestingly, the turning off of some globin genes in our transformed erythroblasts seems to be the inverse of the situation described by Groudine and Weintraub (34,35) for chicken embryo fibroblasts in which globin genes are activated by infection with RNA tumor viruses.
2) or we may assume that the in vivo target cell for the transforming virus is an early erythrocyte progenitor cell of embryonic type, in which some specific genes such as globin are activated but not expressed (36) and in which the methylation pattern of the globin gene region would be different from mature red blood cells.

The theory that transformed cells are blocked at an early stage of differentiation, suggested previously by us (1) as well as by others (37,38), has gained further support by the recent work of Gazzolo et al. (39).

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