Transgene expression in *Xenopus* rods

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Abstract The photoreceptors of the vertebrate retina express a large number of proteins that are involved in the process of light transduction. These genes appear to be coordinately regulated at the level of transcription, with rod- and cone-specific isoforms (J. Hurley (1992) J. Bioenerg. Biomembr. 24, 219–226). The mechanisms that regulate gene expression in a rod/cone-specific fashion have been difficult to address using traditional approaches and remain unknown. Regulation of the phototransduction proteins is medically important, since mutations in several of them cause retinal degeneration (P. Rosenfeld and T. Dryja (1995) in: Molecular Genetics of Ocular Disease (J.L. Wiggs, Ed.), pp. 99–126, Wiley-Liss Inc.). An experimental system for rapidly producing retinas expressing a desired mutant would greatly facilitate investigations of retinal degeneration. We report here that transgenic frog embryos (K. Kroll and E. Amaya (1996) Development 122, 3173–3183) can be used to study cell-specific expression in the retina. We have used a 5.5 kb 5′ upstream fragment from the *Xenopus* principal rod opsin gene (S. Batni et al. (1996) J. Biol. Chem. 271, 3179–3186) controlling a reporter gene, green fluorescent protein (GFP), to produce numerous independent transgenic *Xenopus*. We find that this construct drives expression only in the retina and pineal, which is apparent by 4 days post-nuclear injection. These are the first results using transgenic *Xenopus* for retinal promoter analysis and the potential for the expression in rod photoreceptors of proteins with dominant phenotypes.

Key words: Gene expression; Photoreceptor; Rhodopsin; Promoter; Retina; *Xenopus laevis*

1. Introduction

The *Xenopus* retina is an excellent model system for studies of photoreceptor function and dysfunction. The retina contains approximately equal numbers of rods and cones which express a number of genes involved in phototransduction [1,5]. There are at least three spectrally distinct cones and two rods [6,7]. The principal or green-absorbing rod expresses an opsin that is orthologous to the rhodopsins in other vertebrates and there is an additional blue-absorbing rod comprising less than 5% of the rod population. All photoreceptors are large and amenable to physiological [8] and cell biological [9] techniques. *Xenopus* retinae have been particularly valuable because they can be cultured in vitro for several days while maintaining many aspects of normal physiology [10]. A fundamental limitation with this and other organisms has been the lack of a suitable system for genetic analysis of the proteins involved in photoreceptor function because no efficient method for introduction of altered genes has been available for the terminally differentiated photoreceptors. Transgenic mice and more recently pigs have in part served this role, but are expensive and time consuming to produce. Furthermore, rod-specific promoters that target exclusively to rods but not to cones have not yet been characterized. Finally, it should now be possible to rapidly introduce dominant mutants into rod cells to examine effects on rod morphogenesis, phototransduction and maintenance of the retinal structure. It will be particularly interesting to see if rhodopsin mutations that cause retinitis pigmentosa [2] will also disturb the *Xenopus* retina.

Recently a new method for producing transgenic *Xenopus* embryos suitable for experimental analysis within a few days has been described [3]. This approach utilizes restriction enzyme-mediated integration into permeabilized sperm nuclei whose chromatin has been partially decondensed by incubation with interphase egg extract. Treated nuclei are microinjected into eggs to produce the embryo, some of which incorporate the desired plasmid into the chromosomes.

2. Materials and methods

2.1. DNA constructs

The pXOP-GFP expression plasmids were constructed as follows. The pEGFP-N1 vector (Clontech) was digested with *Vsp*I and *Nhe*I to remove the CMV promoter, filled in using Klenow and reclosed using intramolecular ligation to produce pEGFP(-). A 5.5 kb *Bam*HI fragment from the *Xenopus* principal rod opsin gene [4] was cloned upstream of the EGFP coding region to produce the reporter plasmid pXOP(−5500/+41)GFP. A smaller fragment of the *Xenopus* principal opsin promoter, containing nucleotides −508 to +41, was cloned upstream of EGFP coding region in pEGFP(-) as a *Pst*I/*Bam*HI fragment, pXOP(−508/+41)GFP. DNA was purified using the Qiagen protocol, digested with *Xho*I to linearize the plasmid and purified after digestion (GeneClean, Bio101, Inc.), with final elution in water.

2.2. Production of transgenic Xenopus

Transgenic *Xenopus laevis* embryos were produced using restriction enzyme-mediated integration as described [3] except that incubation of the sperm nuclei/DNA/*Xho*I mixture with the interphase egg nuclear extract was carried out for 3 min. Normal neurulae were selected and cultured in 0.1×MMR (1×MMR: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 5 mM Na-HEPES pH 7.4) at 18–20°C until approximately stage 41 (3–4 days), at which point non-mosaic green fluorescence above the weak background fluorescence from the yolk could be observed in the eye. Tadpoles were maintained in 0.1×MMR until metamorphosis (∼3 months at this temperature).

2.3. Southern analysis

Stage 52 tadpoles (2 months old) with strong fluorescence from the

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eye were frozen in liquid N_2 and genomic DNA was prepared as described [20]. DNA isolated from adult testes and from tadpoles produced by in vitro fertilization served as controls. Five μg DNA was digested with 20 units *Bam*HI for 20 h and then electrophoresed on 0.7% agarose gels. Two probes for Southern blotting were used: the *Pst*I-*Bam*HI fragment from the *Xenopus* rhodopsin promoter (−508/+41) and the *Eco*RI-*Not*I fragment from pEGFP(-) that contained the EGFP coding region. Both probes were labeled with [^{32}P]dATP using the random primer method [21]. Blots had final washes in 0.1×SSC at 55°C, were exposed to storage screens and quantitated using a phosphorimager (Applied Dynamics).

2.4. Microscopy

Transgenic embryos were selected using a stereo dissecting microscope (MZ12 Leica) equipped with a fluorescence module having an enhanced GFP filter cube (Kramer Scientific). Tadpoles were immobilized using tricaine and photographed under direct and fluorescent illumination. Photographic slides were scanned and figures produced using Photoshop (Adobe). For whole mount and cryosections, embryos were fixed overnight in phosphate-buffered 4% paraformaldehyde. Frozen sections (10–15 μm thickness) were stained with B630N [15], an anti-rhodopsin monoclonal antibody. Antibody binding was detected with rabbit anti-mouse IgG coupled to indocarbocyanine (Cy3, Jackson Immunoresearch Laboratories). Confocal images were obtained using a slit scanner instrument (Meridian Insight). Phase images were obtained by turning on the tungsten-halogen lamp during the confocal scan. Confocal images are all pseudocolored.

3. Results and discussion

We have produced transgenic *Xenopus* using the principal rod opsin promoter [4] driving reporter gene expression. Previously, we had characterized the 5.5 kb 5′ upstream fragment and shown that this sequence had transcriptional activity in transient transfections of *Xenopus* embryo heads and injected blastomeres [4]. For producing transgenic embryos, the GFP reporter plasmid (Fig. 1A) was constructed, linearized outside the promoter-GFP region and used to microinject eggs [3]. Approximately 1–10% of the injected eggs produced viable neurulae, the major source of variability being the female egg donor. When the embryos were allowed to develop for 3–4 days post-nuclear injection to a stage when the rods had begun differentiating [11,12] and expressing rhodopsin [13], 20–50% of them could be seen to have green fluorescence emanating from the eye when illuminated with bright blue light (Fig. 2). Prior to development of the pigment in the retinal pigment epithelium (RPE), the fluorescence was also visible by viewing from the dorsal surface (not shown). In addition to the ocular fluorescence, a signal could also be observed from the pineal (Fig. 2f). The appearance of the fluorescence was punctate and consistent with expression in the pinealocytes, which have been suggested to express rhodopsin [14]. In more than 50 embryos, fluorescence was never observed outside the eye and pineal. Thus, the promoter fragment is sufficient for the correct tissue-specific expression of GFP. Moreover, the expression was stable through metamorphosis and in juvenile frogs (Fig. 2g).

Southern analysis was performed to determine the number of copies and integration sites. Digestion of genomic DNA with *Bam*HI followed by hybridization with an opsin promoter probe produced the expected 5.5 kb fragments in control and GFP-expressing tadpoles (Fig. 1B). However, the intensity of hybridization was significantly stronger in the transgenics, indicating additional copies of the opsin promoter were in the GFP-expressing animals. Quantitation of the signal by phosphorimager indicated a 6.7- and 15-fold increase

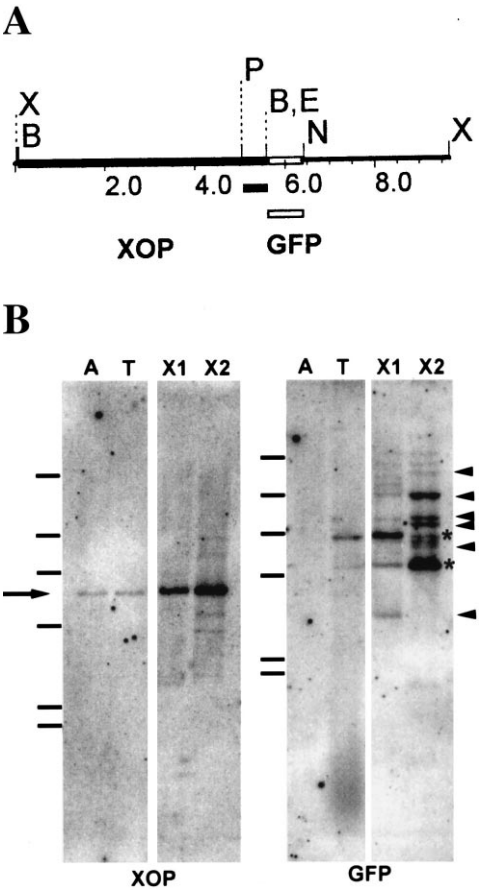

Fig. 1. Integration of rhodopsin promoter-GFP construct into the *Xenopus* genome. A: Map of the transgene construct pXOP(−5500/+41)GFP. On the mapline, the 5.5 kb *Xenopus* principal rod opsin promoter is shown as a solid box and the GFP coding region as an open box. Below the line are shown the probes for Southern analysis. The size marks are in kb and enzyme sites are shown: X, *Xho*I; B, *Bam*HI; P, *Pst*I; N, *Not*I; E, *Eco*RI. B: *Bam*HI-digested DNA from 2 month old tadpoles expressing GFP in the eye (X1, X2), control adult (A) and control tadpole (T) were analyzed by Southern blot using XOP (left) or GFP (right) probes. Arrow indicates the position of the endogenous 5.5 kb *Bam*HI XOP fragment, arrowheads indicate major bands specific to the GFP-expressing tadpoles and asterisks indicate non-specific hybridizing species found only in tadpole DNA. The size markers were λ *Hin*dIII digest in kb: 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0.

in hybridization for the two GFP-positive tadpoles. This corresponds to 12 and 28 additional copies per cell, using two copies of the endogenous principal rod opsin gene [4] for normalization. Most of the additional hybridization in the GFP-expressing tadpoles was found as a 5.5 kb band. Since *Xho*I was used to linearize the plasmid and is 42 bp from *Bam*HI, it appears that extensive rearrangements or deletions are not occurring during integration of this plasmid. In order to determine the number of integration sites, a GFP probe was used for Southern analysis. Numerous (6–10) bands specific for the GFP-expressing tadpole DNA were observed, as well as two cross-hybridizing bands found in variable amounts in all tadpoles (Fig. 1B). These results show that multiple copies of the plasmid have integrated into several places in the tadpole genome.

In order to determine the cellular localization of expression from the transgene, whole mounted retinae were examined.

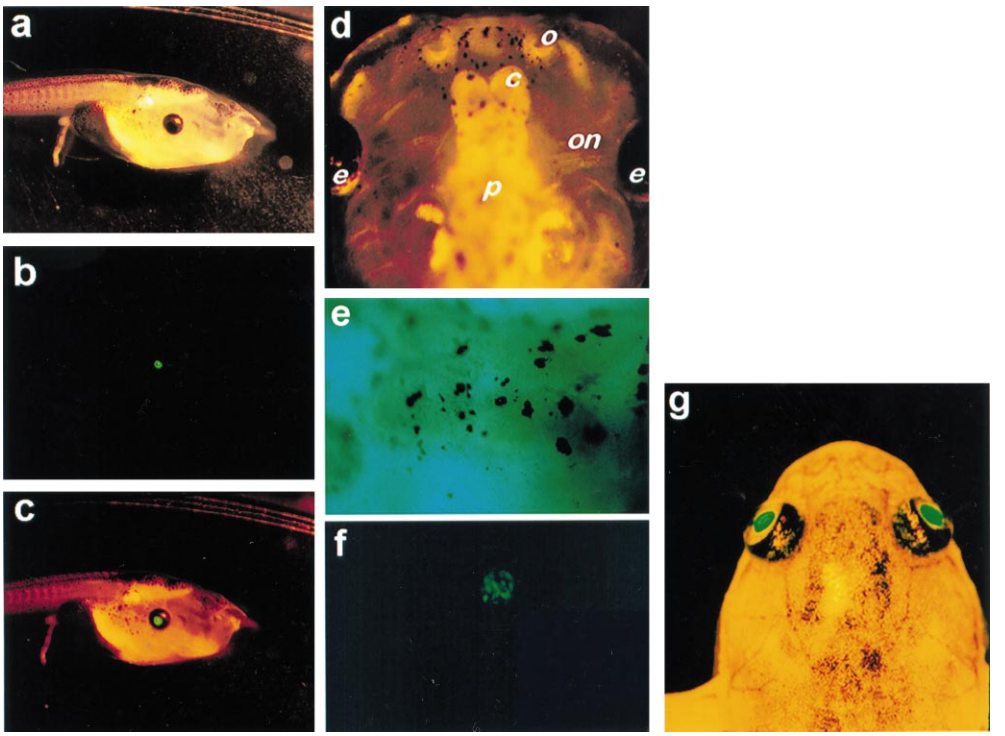

Fig. 2. Lateral view of a transgenic tadpole (stage 51) under bright field illumination (a), blue light (b) or both (c, the tungsten lamp was dimmed compared to a). The bright green fluorescence from the transgene is seen in the eye. d: Dorsal view of a transgenic tadpole (stage 48) under bright field illumination. The position of the eyes (e), optic nerve (on), olfactory organ (o), cerebral hemispheres (c) and pineal (p) are indicated. A higher magnification image of the pineal under bright field (e) and blue light (f). GFP-specific fluorescence is seen as a punctate pattern in pineal. g: The anterior portion of a transgenic juvenile frog illuminated with both tungsten lamp and blue light.

From the whole eye (Fig. 3a, b), the dioptic pattern and fluorescence around the lens and in the embryonic fissure indicates that the fluorescence emanates from the back of the eye. This is confirmed in a whole mount which shows that the GFP-expressing cells are photoreceptors. They fluoresce most strongly in the inner segment, but also in the outer segment and synaptic terminal (Fig. 3c, d). Fluorescence was not seen in other layers of the retina or in the RPE.

To identify the photoreceptor cell type(s) that express GFP, confocal microscopy was used in conjunction with a mono-

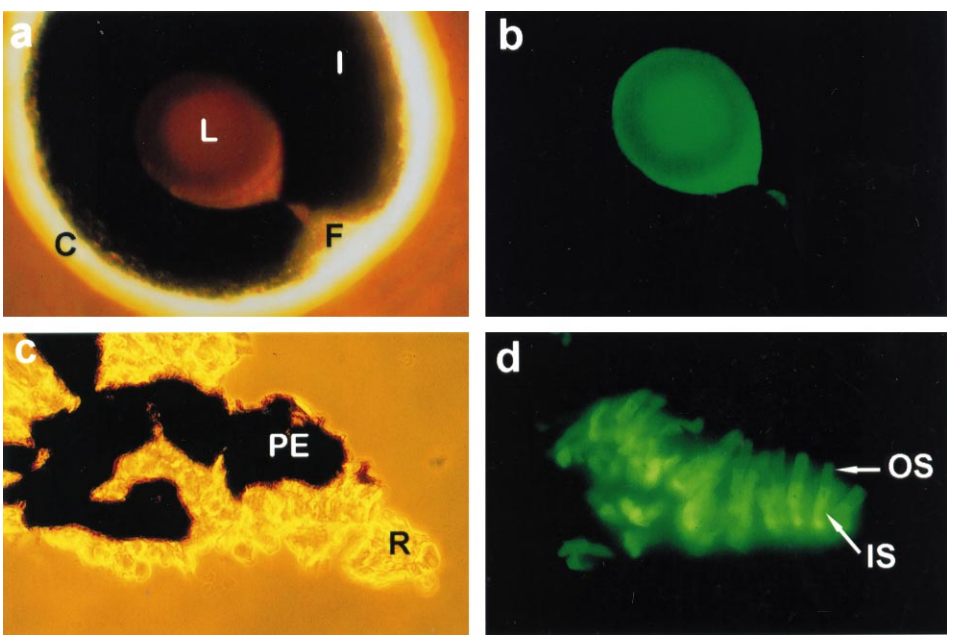

Fig. 3. An enucleated eye from a transgenic tadpole illuminated under bright field (a) or blue light (b). The position of the lens (L), iris (I), choroid (C) and embryonic fissure (F) which is not closed at this stage are indicated. A piece of whole mounted retina from the same eye is shown under bright field (c) and blue light (d). The rod outer (OS) and inner (IS) segments are indicated.

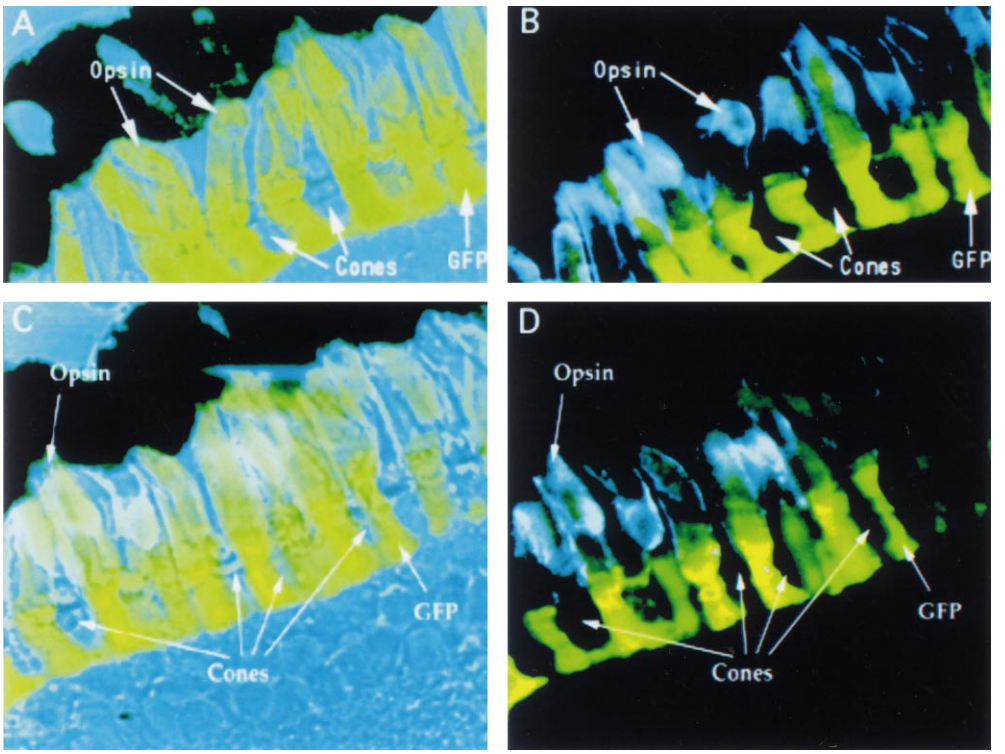

Fig. 4. Confocal images of a retina from a transgenic tadpole produced using pXOP(−5500/+41)GFP. Two separate sections are shown (A and B, C and D). The images under bright field (A, C) were obtained with both bright field and blue light, so the GFP fluorescence (green) is visible in these images. Sections were also viewed for fluorescence from GFP (green) and Cy3 which revealed B630N rod opsin staining (blue) (B, D). Cones are indicated, as well as opsin- and GFP-positive rods.

clonal anti-opsin antibody [15]. Prior work has shown that this antibody (B630N) fails to cross-react with cones or with the less abundant blue-absorbing rod [16,17]. The GFP fluorescence was found only in rod cells which were positive for principal rod opsin by antibody labeling (Fig. 4). There was a one-to-one correspondence between rods positive for B630N reactivity and GFP fluorescence, while cones did not express GFP. Thus, the expression from the 5.5 kb fragment was specific for the abundant principal rod. This is in contrast to mammalian rhodopsin promoters. In transgenic mice, promoter fragments of 2.1 kb (bovine) or 4.4 kb (mouse) drive expression in both rods and cones [18,19]. In order to obtain rod-specific expression in transgenic mice, it was necessary to use an 11 kb fragment containing the mouse structural gene and surrounding 5′ and 3′ sequences [19].

A smaller fragment of the opsin promoter (−508/+41), which has been shown to have similar promoter activity as the 5.5 kb fragment in transiently transfected embryos (Batni and Knox, unpublished), also targeted GFP expression exclusively in rods (data not shown). Thus, the *Xenopus* principal rod opsin promoter is more compact than mammalian promoters, and contains the elements specifying rod-specific transcription within the immediate 5′ upstream sequences.

The experiments reported here demonstrate the rod-specific expression of GFP in transgenic tadpoles. The production of tadpoles is rapid (4 days), numerous independent lines can be generated and the expression is specific for the desired cell type. Unlike previous techniques for introducing genes into *Xenopus*, this approach generates non-mosaic reporter gene expression, i.e. in all rods in the retina. Determining *cis*-regulatory elements necessary for rod- and cone-specific expression should be rapidly achievable. In addition, the large number of cones in this retina make it particularly suited for studies of cone biology using transgenic techniques.

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References

[1] Hurley, J. (1992) J. Bioenerg. Biomembr. 24, 219–226.
[2] Rosenfeld, P. and Dryja, T. (1995) in: Molecular Genetics of Ocular Disease (Wiggs, J.L., Ed.), pp. 99–126, Wiley-Liss Inc., New York.
[3] Kroll, K. and Amaya, E. (1996) Development 122, 3173–3183.
[4] Batni, S., Scalzetti, L., Moody, S.A. and Knox, B.E. (1996) J. Biol. Chem. 271, 3179–3186.
[5] Saxen, L. (1954) Ann. Acad. Sci. Fenn. Ser. A 23, 5–93.
[6] Zhang, J., Kleinschmidt, J., Sun, P. and Witkovsky, P. (1994) Vis. Neurosci. 11, 1185–1192.
[7] Rohlich, P., Szel, A. and Papermaster, D.S. (1989) J. Comp. Neurol. 290, 105–117.
[8] Hassin, G. and Witkovsky, P. (1983) Vis. Res. 23, 921–931.
[9] Besharse, J. and Dunis, D. (1983) Science 219, 1341–1343.
[10] Besharse, J. and Iuvone, M. (1983) Nature 305, 133–135.
[11] Hollyfield, J. and Rayborn, M. (1979) Invest. Ophthal. Vis. Sci. 18, 117–132.
[12] Holt, C.E., Bertsch, T.W., Ellis, H.M. and Harris, W.A. (1988) Neuron 1, 15–26.
[13] Saha, M. and Grainger, R. (1993) Mol. Brain Res. 17, 307–318.
[14] Korf, B., Rollag, M. and Korf, H. (1989) Cell Tissue Res. 258, 319–329.
[15] Adamus, G., Zam, Z., Arendt, A., Palczewski, K., McDowell, J. and Hargrave, P. (1991) Vis. Res. 31, 17–31.

[16] Besharse, J. and Wetzel, M. (1995) J. Neurocytol. 24, 371–388.
[17] Stiemke, M., Landers, R., Al-Ubaidi, M., Rayborn, M. and Hollyfield, J. (1994) Dev. Biol. 162, 169–180.
[18] Gouras, P., Kjeldbye, H. and Zack, D. (1994) Vis. Neurosci. 11, 1227–1231.
[19] Woodford, B., Chen, J. and Simon, M. (1994) Exp. Eye Res. 58, 631–635.
[20] Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. and Struhl, K. (1993) Current Protocols in Molecular Biology, pp. 2.9.1–2.9.20, J. Wiley, New York.
[21] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.