Isolation of Extrachromosomal Elements by Histone Immunoprecipitation

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ABSTRACT

Here, we describe a gentle and effective method for the rapid and reproducible isolation of histone-bound extrachromosomal DNA molecules called extrachromosomal elements (EEs). This method facilitates the harvest of a specific population of EEs following their isolation from cultured cells, primary tissues, and tumor cells. Active EEs are bound to histone proteins, and these histone-bound EEs carry actively transcribing genes such as c-myc. Our method exploits the presence of histones on EEs and serves as a first-step purification procedure, allowing for the cloning or multivariant analysis of an immunopurified sample of EEs. We isolated EEs from 4-hydroxytamoxifen (4-HT)-activated Myc-ERTM-regulatable Pre-B ABM cells. Following one round of immunoprecipitation, we demonstrate the purification of histone-bound EEs. We confirmed that our purification enriched for EEs that carry genes by fluorescent in situ hybridization of EEs (FISH-EEs), and we probed non-enriched and immunopurified EEs with a dihydrofolate reductase (DHFR) cDNA probe that is known to detect extrachromosomal amplification in Myc-activated cells. We demonstrate the enrichment of immunoprecipitated DHFR-containing extrachromosomal DNA molecules.

INTRODUCTION

Gene amplification is one of the most common phenomena found in biology. It occurs in virtually all types of organisms (1,21,22). While amplification is rarely found in normal cells (4,18,27,28,31), it is a significant factor in human cancers because it has been clearly associated with tumor initiation and progression (3,26). Gene amplification can be intrachromosomal and can be visualized as homogeneously staining regions (HSRs), or it can be found extrachromosomally in the form of double minutes (DMs) (6,9,22,23), also referred to as extrachromosomal elements (EEs). In many cases, EEs have been shown to carry oncogenes (12) and drug-resistant genes (20,29). Recently, Wiener et al. (30) described a population of *c-myc/IgH*-carrying EEs that were able to actively transcribe *c-myc* mRNA and functionally replace a Myc-activating chromosomal translocation. These results indicate that EEs are likely to contribute to tumorigenesis and neoplasia. For this reason, EEs require further detailed analysis.

The study of gene amplification, rearrangement, or deletion has evolved from standard methods such as classical cytogenetics, Southern blotting, and dispersed cell assays, to more sophisticated and powerful methods such as fluorescent in situ hybridization (FISH), spectral karyotyping (SKY), and comparative genomic hybridization (CGH). All of these advanced technologies can be used for the characterization of EEs. However, each technology is currently limited in its capacity to be used in the study of Hirt-extracted EEs because this first-stage preparation consists of a het-

erogeneous population of EEs carrying repetitive DNA sequence motifs and extrachromosomal genes (7).

Recently, greater advances have been made in the characterization of amplified genes, specifically EEs. Each of the more sophisticated technologies currently used can be applied to greater potential following the additional purification of EE samples. This includes FISH-EEs (13), Southern and pulsed field gel electrophoreses, mRNA FISH (25), and creation of EE libraries. Recently, Wiener et al. (30) demonstrated that functional EEs carried active histone proteins. We have used the presence of histones on functional EEs to develop a method enabling the isolation of EEs that carry genes. To facilitate the isolation of functional EEs extracted from tissue culture cells or clinical samples, we have further adapted the Hirt protocol (10), which was originally designed to isolate polyoma virus particles. Preparations of EEs are frequently contaminated with varying amounts of genomic DNA and/or apoptotic DNA fragments (19). The contamination of EEs with genomic and apoptotic DNA was addressed by Gaubatz and Flores (8), who described the use of exonuclease III, an enzyme that removes linear DNA molecules from these preparations. Exonuclease III also digests open and nicked circular extrachromosomal DNA molecules, potentially eliminating some of the EEs. Therefore, the use of this enzyme may compromise the isolation of a true and representative population of potential transcriptionally or replicationally active EEs from a cell.

There are several advantages to immunopurifying EEs before their analysis. First, this immunoprecipitation

method can be used as a primary post-extraction purification step following the Hirt isolation (10) of EEs. This immunopurification method allows for a number of analytical procedures to be conducted on a pure and heterogeneous population of EEs without genomic contaminants derived from cultured cells, primary tissue, or tumor samples. In our experiments, immunopurified samples showed an enriched population of EEs that carry *DHFR* gene sequences and are free of genomic contaminants. Second, this purification method allows further experimentation with a greater degree of resolution. In the past, classical karyotype analysis was only able to show that EEs were present in the karyotype, although the sequence content of these EEs was not characterized. Recently, FISH-EEs (13) was successfully applied to test for the presence of genes on freshly isolated EEs. Using the ability to immunopurify EEs before performing FISH-EEs, it is now possible to identify potentially active genes that are co-localized on a selected, but representative population of potentially functional EEs.

SKY has proven invaluable in visually identifying chromosomal rearrangements in tumor cells and in identifying the presence of EEs and their chromosomal origins (2). SKY analysis of EEs can be refined by probing immunopurified EEs specifically for histone-carrying and potentially active genes that are amplified or deleted from whole chromosomes, as opposed to the SKY analysis of all extrachromosomal DNA that is not necessarily associated with active chromatin.

CGH is a technology that is capable of detecting amplifications and deletions in genomic DNA (11). CGH can also be used to examine extrachromosomal DNA. The analysis of CGH on immunopurified EEs in combination with CGH on genomic DNA can resolve the degree of amplification found on EEs and determine whether certain EEs are extrachromosomal amplification products derived from HSRs or the products of a chromosomal deletion. Thus, CGH can be used more precisely when only potentially active populations of EEs are compared. The isolation of large quantities of potentially active EEs using the immunoprecipita-

tion method also facilitates the use of more traditional approaches such as Southern and pulsed field analyses of extrachromosomal DNA. Finally, the immunopurification of EEs expedites the isolation of sufficient quantities of purified and potentially active EEs, allowing for the creation of enriched EE libraries, which previously had not been possible.

MATERIALS AND METHODS

Cell Culture

A mouse Pre-B ABM cell line was used for the generation and isolation of extrachromosomal DNA purification. This is a Myc-regulatable cell line that carries a Myc-ERTM that is activated by the addition of 100 nM 4-hydroxytamoxifen (4-HT) (Sigma, Winston, ON, Canada). For simplicity, non-activated Pre-B cells are referred to as Pre-B-, and 4-HT-activated Pre-B cells are referred to as Pre-B+. The origin and culture conditions of Pre-B cells have been described (14,16).

Isolation of EEs

We isolated EEs from Pre-B+ cells following activation (14). Our procedure for EE isolation is based on the Hirt protocol (10) and modified as previously described (13). The Hirt extract contains the bulk of the EEs and total cellular RNA, but it may also be contaminated with small, linear fragments of genomic DNA and apoptotic DNA fragments that have been estimated to comprise 0.5%–1.0% of the total amount of EEs isolated (19). Following isolation, all of the manipulations of EEs were performed in sterile, siliconized, microcentrifuge tubes (Fisherbrand®; Fisher Scientific, Pittsburgh, PA, USA).

Determination of Apoptotic or Genomic DNA Contamination in Non-Immunopurified Pre-B- and Pre-B+ Cell-Derived EEs

Preparations of EEs were made from Pre-B- and Pre-B+ cells in triplicate. DAPI (Sigma) (1 µg/mL in PBS) was used to counterstain the EEs and any genomic DNA contaminants. The

samples were then visualized and photographed using a Zeiss® Axio-planTM 2 microscope (Carl Zeiss, Ottawa, ON, Canada) under a 63× oil-immersion objective and a UV filter. The images were acquired using Northern EclipseTM 5.0 software (Empix Imaging, Mississauga, ON, Canada) and a model XC75 Sony® CCD camera. The percentage of contaminating DNA was assessed in each of the preparations by manually counting and totaling EEs and signals from contaminating apoptotic or genomic DNA (19). We found that Pre-B- and Pre-B+ cell-derived EE preparations carried 10.8% ± 2.5% and 12.1% ± 4.9% apoptotic or genomic DNA contamination, respectively.

Purification of Histone-Containing Extrachromosomal DNA Molecules

We pretreated the protein G sepharose beads (Amersham Pharmacia Biotech, Baie d'Urfé, PQ, Canada) used for the immunopurification of the EEs. We washed 1 µg protein G sepharose beads in 5 mL buffer A [100 mM KCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT (all purchased from Sigma), and 1 mM Pefabloc® SC proteinase inhibitor (Roche Diagnostics, Laval, PQ, Canada)] by placing them on a rotating platform for 10 min at room temperature. The beads were centrifuged at 13 250× *g* for 10 min at room temperature, and the supernatant was removed and discarded. This washing procedure was performed three times. The nonspecific binding sites on 40 µL beads were blocked by resuspending the beads in 300 µL buffer B [buffer A + 4% (w/v) BSA (Sigma)]. Figure 1 shows the binding of the sheep anti-core histone antibody to the protein G sepharose beads.

Immunopurification of EEs was used to isolate the Hirt-extracted, histone-bound EEs from the impurities found in the Hirt-extracted EEs sample. Freshly extracted EEs were dialyzed against 1× TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) overnight at 4°C. The EEs were then incubated in 500 µL buffer B on a rotating platform for 10 min at room temperature. We added 1 µg sheep polyclonal anti-core histone antibody per microgram of EEs and incubated the mixture for 30 min at room

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temperature on a rotating platform. The blocked beads were then added to the antibody-treated EEs and incubated overnight on a rotating platform at room temperature. Following this incubation, the EE/beads were centrifuged at $13\,250\times g$ for 10 min at room temperature to remove any unbound material. The EE/beads were then washed three times with buffer A to remove any residual unbound material. We eluted the bound histone-containing EEs by adding 300 μL 100 mM glycine, pH 2.3 (Sigma) to the beads and mixing them gently by inverting the tube 5–10 times. The tube was centrifuged at $13\,250\times g$ for 10 min at room temperature, and the supernatant (eluate) was removed and collected. This eluate was immediately neutralized to pH 7.2 using 1 M Tris, pH 8.0. The elution step was repeated once more, and then the two collected eluates were pooled together. The sample was concentrated to approximately 40 μL by roto-evaporation.

Analyses of Immunopurified EEs

The following protocol allows the fixation of the EEs onto glass microscope slides and ensures that the EEs are contained within a small area. Briefly, 40 μL of Hirt extract or immunopurified and concentrated EEs were diluted in a 1:1 ratio in a fixative solution [freshly prepared 3:1, methanol (Fluka, Winston, ON, Canada) and acetic acid] and then delivered onto slides (O. Kindler, Germany) precooled for 60 s on dry ice. The slides were then immediately moved onto a slide warmer (37°C). The fixation procedure was completed as described previously (13).

We visualized non-immunopurified and immunopurified Hirt-extracted EEs by immunohistochemical staining (14). For this procedure, we used a sheep anti-core histone antibody diluted 1:400 in lamb serum (Life Technologies, Burlington, ON, Canada), and incubated for 30 min at room temperature. This was followed by incubation with a secondary antibody, a fluorescein isothiocyanate (FITC)-conjugated, donkey anti-sheep IgG antibody (Sigma) diluted 1:400 in lamb serum (Life Technologies, Burlington, ON, Canada), and incubated for 30 min at room temperature. Immunofluorescent analysis of histone-bound EEs was performed as previously de-

scribed (14), the only modification being the omission of the permeabilization step. Anti-bleach (15) was added to preserve the fluorescence of the sample and to function as a mount for the coverslip. We analyzed the immunostained EEs using an Axioplan 2 microscope under a $63\times$ oil-immersion objective and a UV filter. The images were acquired using Northern Eclipse 5.0 software and a model XC75 Sony CCD camera. The software's adaptive thresholding tools were used to remove all dots from the DAPI-stained images that were less than or equal to 5×5 pixels in size when visualized using a $63\times$ oil-immersion objective lens and a $0.63\times$ adapter.

FISH-EEs (13) was performed on Hirt-extracted EEs and on immunopurified EEs using a *DHFR* probe (15). We analyzed the EEs using an Axiophot[®] microscope (Carl Zeiss) under a $100\times$ oil-immersion objective, a $1\times$ magnification adapter, and a UV filter. The images were acquired using IPLab[®] software (Scanalytics, Fairfax, VA, USA) and a Photometrics (CH250/a) CCD

camera equipped with a KAF[®]-1400–1450 sensor chip, 1317×1035 pixels (Eastman Kodak, Rochester, NY, USA).

RESULTS

The isolation of the histone-bound population of extrachromosomal DNA extracted from Pre-B+ cells (10) was performed by immunoprecipitation. Pre-B+ cells contain a 4-HT-responsive Myc-ER construct and have been activated with 4-HT to overexpress c-Myc (14). This induces the activation of EEs from c-Myc-activated Pre-B+ cells (14,16). These EEs were isolated by Hirt extraction followed by the immunoprecipitation of histone-bound EEs.

Binding of Anti-Core Histone Antibody to Protein G Sepharose Beads

Sheep anti-core histone antibody was bound to protein G sepharose beads according to the manufacturer's instructions. Briefly, the anti-histone antibody

Figure 1. Binding and elution of sheep anti-core histone antibody to protein G beads. Lane 1 was loaded with 4% (w/v) BSA. Lane 2 was loaded with 20 μg sheep anti-core histone antibody. Lane 3 contains unbound sheep anti-core histone antibody from the protein G sepharose beads. Lanes 4–6 were loaded with washes 1–3 in which the protein G sepharose beads were washed with buffer A. Lane 7 contains sheep anti-core histone antibody following elution with 100 mM glycine, pH 2.3, and neutralization with Tris, pH 8.0. The black arrow indicates bands corresponding to sheep anti-core histone antibody following elution from protein G sepharose beads using 100 mM glycine, pH 2.3, and immediate neutralization with 1 M Tris, pH 8.0.

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was eluted from the protein G sepharose beads by incubation in 100 mM glycine buffer, pH 2.3. The eluate was immediately neutralized to pH 7.2 using 1 M Tris buffer, pH 8.0. Figure 1 shows the successful binding and elution of the sheep anti-core histone antibody from the protein G sepharose beads.

Immunostaining Shows Enrichment of Histone-Bound EEs following Histone Immunoprecipitation of EEs (HIP-EEs)

Figure 2, panels a–f, shows immunohistochemical staining for the presence of histone proteins on EEs. Non-immunopurified EEs (panels a–c) are compared with immunoprecipitated EEs (panels d–f). In this figure, the large white arrows indicate representative co-localized DAPI and FITC signals, and the small white arrow indicates a representative FITC signal that does not co-localize with a light blue DAPI signal (Figure 2f). Figure 2, panels a–c, shows the relative proportion of histone-bound EEs in a sample of nonpurified EEs. Figure 2a shows a large population of EEs, indicated by pale blue DAPI signals. Figure 2b shows light yellow or white signals from FITC-conjugated donkey anti-sheep antibody, indicating the presence of EEs bound by histone proteins. Figure 2c is the overlay of panels a and b in which light yellow FITC signals are overlaid with the pale blue DAPI-stained EEs. These overlaid signals are visible as distinct, light yellow or white dots. In the antibody-purified population (Figure 2, panels d–f), we observed an enrichment in histone-bound EEs. Figure 2d shows a sparse population of DAPI-stained extrachromosomal DNA, seen as blue dots. Figure 2e shows an equally sparse population of pale yellow dots generated by the FITC-conjugated donkey anti-sheep antibody and corresponding to extrachromosomal DNA after immunopurification of histone-bound EEs. Figure 2f illustrates the overlay of panels d and e. In Figure 2f, the majority of DAPI signals co-localize with FITC signals and appear as pale blue dots (see large white arrows). Figure 2f also shows FITC signals where there is no co-localization with DAPI signals (see small white arrow). The likely reason for this is that

DAPI intercalates into the DNA, but its signal was not amplified further. In contrast, the sheep anti-core histone antibody was amplified by the FITC-conju-

gated donkey anti-sheep secondary antibody, giving a greater relative signal intensity in comparison to the weaker DAPI signal intensity.

Figure 2. Immunoprecipitation of EEs with anti-core histone antibody isolates histone-bound EEs. Panels a–f show the relative proportion of histone-bound EEs in nonpurified EEs from Pre-B+ cells. (a) DAPI-stained nonpurified Hirt-extracted EEs. (b) Anti-core histone antibody immunostaining of EEs (green dots). (c) Overlays of panels a and b. (d–f) Relative proportion of histone-bound EEs in purified EEs. (d) DAPI-stained immunopurified Hirt-extracted EEs. (e) Anti-core histone antibody immunostaining of immunopurified EEs. (f) Overlays of panels d and e. Large white arrows in panel f indicate co-localized DAPI and FITC signals. The small white arrow indicates a representative FITC signal without a co-localized DAPI signal (see Results for details).

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The data show that there are fewer DAPI signals in the immunopurified sample (Figure 2, panels d–f), indicating fewer EEs in the immunopurified sample compared to the non-immunopurified EEs. However, out of the EEs isolated by immunopurification, the majority is co-localized with bright yellow or white signals indicative of histone protein. This demonstrates an enrichment of histone-bound EEs.

FISH-EEs Show Enrichment of *DHFR* Sequences on EEs Following HIP-EEs

To assess the value of this EE immunopurification method, we assayed for the enrichment of an extrachromosomally amplified gene. It had been demonstrated previously that c-Myc deregulation results in amplification of *DHFR* in mouse, rat, hamster, and human cell lines (17). By the FISH-EEs method, we showed that *DHFR* was present on extrachromosomal DNA from Pre-B+ cells (13). We hypothesized that the enrichment of the Hirt-extracted EEs by our immunopurification method should increase the relative ratio of *DHFR*-containing EEs when comparing non-immunoprecipitated against immunoprecipitated EE sample populations.

Figure 3, panels a–f, illustrates the comparison of *DHFR*-containing EEs from non-immunopurified EEs (Figure

3, panels a–c) with *DHFR*-containing EEs from immunopurified EEs (Figure 3, panels d–f). In Figure 3f, large white arrows indicate representative co-localized DAPI and FITC signals, a small white arrow indicates a representative FITC signal that does not co-localize with a DAPI signal, and an open arrow indicates a representative DAPI signal where there is no co-localized FITC signal. Figure 3, panels a–c, shows the

result of FISH-EEs experiments, where we probed a non-immunoprecipitated population of Hirt-extracted EEs with a digoxigenin (DIG)-labeled *DHFR* cDNA probe. Figure 3a shows a large population of DAPI-stained EEs, shown as pale blue dots. Figure 3b shows a sparse population of EEs detected by a DIG-labeled *DHFR* cDNA probe and amplified by a FITC-conjugated anti-DIG antibody. These signals

Figure 3. Immunoprecipitation of EEs with anti-core histone antibody enriches for EEs that carry *DHFR* sequences. Panels a–c show the relative proportion of non-immunopurified EEs that carry *DHFR* gene sequences. (a) DAPI-stained non-immunopurified Hirt-extracted EEs. (b) EEs probed by FISH-EEs using a DIG-labeled *DHFR* cDNA probe detected by a FITC-conjugated anti-DIG antibody. (c) Overlays of panels a and b. Panels d–f show the relative proportion of immunopurified EEs that carries *DHFR* gene sequences. (d) DAPI-stained immunopurified Hirt-extracted EEs. (e) Immunopurified EEs that carry *DHFR* gene sequences. (f) Overlays of panels d and e. Large white arrows in panel f indicate representative co-localized DAPI and FITC signals. A small white arrow indicates a representative FITC signal without a co-localized DAPI signal. An open white arrow shows an example of EEs in which a light blue DAPI signal is seen without a FITC co-localized signal (see Results for details).

appear as small green, or larger pale yellow dots. The overlay of Figure 3, panels a and b, shows the co-localization of DAPI and FITC signals and appears as pale yellow or white dots. Figure 3, panels a–c indicates a small number of *DHFR* FISH-EEs signals relative to a large number of DAPI signals in the non-immunopurified EE population. Figure 3, panels d–f, shows the results of FISH-EEs experiments conducted with immunopurified EEs. Figure 3d shows that there is a smaller number of DAPI-stained EEs in the population isolated by immunopurification than in the nonpurified sample shown in Figure 3a. These signals appear as pale blue dots. Figure 3e shows a greater number of EEs hybridizing to a *DHFR* probe than in the nonpurified sample (Figure 3b), the signals appearing as pale yellow signals. The overlay of Figure 3, panels d and e, shows a higher proportion of *DHFR*-stained EEs that co-localize to DAPI-stained EEs (Figure 3f). In Figure 3f, large white arrows indicate representative *DHFR* signals that co-localize with DAPI signals. This overlay shows an increased proportion of *DHFR*-containing EEs in comparison to the nonpurified EE sample. A small white arrow in Figure 3f shows a representative FITC signal that does not co-localize with a DAPI signal. As described above, this is likely caused by the relatively poor signal intensity of DAPI in comparison with the amplified *DHFR* signal amplified by a secondary antibody (see Materials and Methods). Figure 3f shows an increase in the number of co-localized DAPI-FITC signals in comparison with the proportion of DAPI-FITC co-localized signals in the nonpurified sample shown in Figure 3c. In Figure 3f, we also observed a number of DAPI signals that did not show co-localization with a FITC signal (see open arrow). These are likely to be histone-containing EEs that do not contain *DHFR* sequences but may contain sequences from other gene families (5,13,14,16).

Overall, these data indicate enrichment in the number of EEs that carry *DHFR* sequences and presumably sequences of other c-Myc target genes such as *ribonucleotide reductase R1* and *R2* genes (13,14), *cyclin D2* (16), and potentially other unidentified genes (5).

DISCUSSION

The immunopurification of the active population of extrachromosomal DNA molecules is advantageous in studying functional EEs because it enriches for a potentially active population of EEs, removing the non-histone-bound and presumably inconsequential population of EEs from the extrachromosomal DNA population. The value of this method as a primary means of isolating potentially functional EEs from a large population of extrachromosomal DNA amplicons was assessed by two methods.

We first determined the percentages of contaminating apoptotic or genomic DNA in each EE preparation. We prepared EEs from non-immunopurified Pre-B- and Pre-B+ cells. Our results indicate the Pre-B- and Pre-B+ had $10.8\% \pm 2.5\%$ and $12.1\% \pm 4.9\%$ genomic DNA contaminant, respectively. Immunoprecipitation of histone-bound EEs helps to eliminate these contaminants.

We examined non-immunopurified and immunopurified EEs by immunostaining for histone protein and by comparing the ratio of DAPI-stained DNA molecules that co-hybridized with the signal from an anti-histone antibody (Figure 2, panels a–f). We saw an enrichment of histone-bound EEs in the immunopurified samples. Nearly all of the EEs in the sample of immunopurified EEs showed co-localization with FITC, indicating that the majority of the EEs in the purified samples contained histone proteins. The results from the immunostaining assay show that this method is successful in isolating histone-bound EEs from a large pool of EEs that do not contain histone protein.

We then assessed the number of EEs that hybridize with a gene of interest, in this case *DHFR* (Figure 3, panels a–f). The experiments show that immunopurified EEs carry a larger relative number of *DHFR* sequences, indicating an overall enrichment of specific extrachromosomal products. Figure 3f shows that although there is an increase in the proportion of EEs that hybridize with a *DHFR* probe, there are also several EEs in which no FITC signal is seen to co-localize with DAPI-stained EEs. This is expected because previous work has shown that a number of genes

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can be found on EEs from Myc-ER-activated mouse Pre-B+ cells. These include *ribonucleotide reductase R1* and *R2* (13,14), *cyclin D2* (16), and *DHFR* (15) sequences. It is likely that there are others as well (5).

In conclusion, we have shown that our method for the immunopurification of EEs is useful as a means of studying extrachromosomal gene amplification phenomena and amplification-mediated expression of oncogenes, drug resistance genes, and potentially others. A body of literature is accumulating describing the role of extrachromosomal DNA molecules in tumor initiation and progression. The immunopurification of EEs is a novel tool that is ideally suited as a first-step purification of EEs for a variety of studies in cultured cells, primary cells, and tumor samples. These analyses and procedures include generating libraries of EEs from cells, analyses of EEs by electron microscopy, FISH-EEs (13), mRNA FISH (25,30), cloning, and Southern and pulsed field analyses.

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