Minichromosome assembly of non-integrated plasmid DNA transfected into mammalian cells

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ABSTRACT

The nucleoprotein structures formed on various plasmid expression vectors transfected into mammalian cells by both the calcium phosphate and DEAE-dextran methods have been studied. We demonstrate by a variety of means that mammalian cells are capable of rapidly assembling non-integrated circular plasmids (both replicating and non-replicating) into typical "minichromosomes" containing nucleosomes with a 190 bp repetitive spacing. Treatment of recipient cells with sodium butyrate for a short period of time (12-16 h) immediately following transfection markedly increased the DNase I digestion sensitivity of the newly assembled plasmid chromatin. Furthermore, minichromosomes isolated from such butyrate-treated cells are depleted in histone H1 and contain highly acetylated forms of histone H4. These findings are entirely consistent with our earlier speculation (Gorman et al., Nucleic Acids Res. 11, 1044; 1983) that appropriate butyrate treatment might stimulate transient expression of newly transfected genes by facilitating their assembly into an "active" type of chromatin structure.

INTRODUCTION

Dynamic and often reversible changes in chromosome structure play an important role in the *in vivo* regulation of gene expression in eukaryotic cells (recently reviewed in 1). Transcriptionally competent chromatin is distinguished from the bulk of inactive nuclear chromatin by a number of characteristics. Active genes appear to be packaged in altered forms of nucleosomes that are more sensitive to digestion by DNase-I (2) and other nucleases than are bulk nucleosomes. Active genes likewise seem to be preferentially associated with certain non-histone proteins, with hyperacetylated forms of histones, and may well be located in specific "domains" of the nucleus that are topologically constrained and perhaps under a certain degree of torsional stress.

In a previous report (7) we established that the transient expression of several different foreign genes carried on plasmid expression vectors introduced by the calcium phosphate transfection technique into a variety of mammalian cell types could be markedly stimulated by short-term treatment of the recipient cells with sodium butyrate. We speculated (7) that one of the possible mechanisms by which butyrate might facilitate the transient expression of such a wide variety of

exogenously supplied non-integrated foreign genes was to facilitate their assembly into an "active" type of chromatin structure. Subsequently, we obtained preliminary evidence supporting such a hypothesis (8). Here we report in detail experiments that demonstrate that both replicating and non-replicating supercoiled plasmid DNAs transfected into mammalian cells can be assembled in the nucleus into nucleosome-containing minichromosomes. Furthermore, we demonstrate that brief butyrate treatment of transfected cells during the initial phases of chromatin assembly not only markedly stimulates the expression of newly introduced foreign genes but also alters several structural features of the newly assembled minichromosomes to resemble those believed to be characteristic of "active" chromatin.

MATERIALS AND METHODS

Cells and Plasmids

Methods used for the routine maintenance of mammalian tissue culture cell lines and for bacterial cells and plasmids have been described (9). Plasmid pSV2cat, containing the bacterial gene coding for chloramphenicol acetyltransferase (CAT) under the promoter control of the "origin" region of SV40 virus (9), was the primary expression vector used in DNA-mediated cell transfection experiments but other plasmids were also employed. These included 1) derivatives of the hybrid pSV2 expression vector (11) containing either the bacterial *E. coli* xanthine-guanine phosphoribosyltransferase (gpt) gene (pSV2gpt) (12), or the Tn5 aminoglycoside phosphotransferase (neo) gene (pSV2neo) (13); 2) the plasmid pRSVcat containing the CAT gene under strong promoter control of the "long terminal repeat" sequence of Rous sarcoma virus (6). Mammalian tissue culture cell lines utilized in transfection experiments were: CV-1 African green monkey kidney cells; COS-1 cells which carry an origin-defective integrated copy of SV40 that expresses T-antigen constitutively and thereby supports the replication of SV40-origin-containing plasmids (17); mouse cell line NIH/3T3.

Plasmid Preparation and Transfection Techniques

Protocols for the high efficiency transformation of mammalian cells employing modifications of the calcium phosphate (CaPO_4) procedure of DNA-mediated transfection have been described in detail elsewhere (9, 19). Transfection of cells by the DEAE-dextran technique followed the procedures of McCutchan and Panago (15) as modified by Sussman and Miller (16) using supercoiled DNA and employing a glycerol shock (18). Methods for the appropriate short-term treatment of newly transfected cells with sodium butyrate to enhance transient expression of plasmid DNA have been described (7). For the plasmids pSV2cat and pRSVcat transient expression was monitored using the enzyme assay for chloramphenicol acetyltrans-

ferase (CAT) activity described by Gorman et al. (9).

Supercoiled plasmid DNAs radioisotopically labeled to high specific activity in vivo were isolated from the mutant *E. coli* K-12 strain W3110 (thy⁻) grown in M9 minimal medium (10) supplemented with 1% glucose, 0.2% cas-amino acids, 2 µg/ml of thymine and either (³H)- or (¹⁴C)-thymidine. Supercoiled plasmid DNAs with specific activities of either 2.3-2.7 x 10⁶ cpm of (³H)/µg or 1.8-2.1 x 10⁵ cpm of (¹⁴C)/µg were obtained.

Nuclear, Nucleosome and DNA Isolation Procedures

Nuclei were isolated from tissue culture cells as described (14). For digestion with micrococcal nuclease (E.C. 3.1.4.7) nuclei were resuspended at a concentration of between 100-200 O.D. 260/ml (5-10 mg/ml of DNA) in a buffer solution (at 4 ° C) containing 25 mM Tris (pH 7.6), 12 mM KCl, 2.5 mM MgCl₂, 5 mM sodium butyrate, 0.5% CaCl₂ and 0.15 mM phenylmethylsulfonylfluoride. Micrococcal nuclease at 0.65 units/1.0 O.D. 260 unit of chromatin was added to the solution and the nuclear suspension immediately transferred to room temperature (22 ° C) and incubated for the times (0-10 min) indicated in the text before termination by addition of 0.1 volume of a 0.5 M EDTA solution and quenching at 4 ° C.

The general methods used for the preparation and purification of micrococcal nuclease released nucleosomes and nucleosomal DNA have been reported elsewhere (14). Centrifugation of the nucleosomal preparations on a 10-30% sucrose gradient was for 18 h at 30,000 rpm in a Beckman ultracentrifuge using a SW41 rotor. Nucleosome repeat length calculations were performed using the method of Shelton et al. (24).

Nucleoprotein complexes of plasmid DNA molecules were isolated as "mini-chromosomes" from cells between 72-144 h after transfection (depending on the experiment) by the method of Fernandez-Munoz et al. (19) with modifications described by Choder et al. (21). Concentrated nuclear extracts containing the minichromosomes were layered on 5-20% sucrose gradients, centrifuged for 2.5 h (4 ° C) at 30,000 rpm in an SW41 rotor, gradient fractions collected and analyzed.

Other Techniques

Digestion of nuclei from transfected cells with pancreatic DNase I (E.C.3.1.4.5) was as described by Wu (3) with slight modifications. Standard recombinant DNA and nucleic acid hybridization procedures were employed (10). DNA separated by electrophoresis in agarose gels was transferred to nitrocellulose (29) and hybridized to appropriate molecular probes labeled to high specific activities (10⁸-10⁹ cpm/µ g) with (³²P)-(α)-dCTP (New England Nuclear) by nick translation *in vitro*.

The replicative state of plasmids in host cell nuclei was assessed by

monitoring the bacterial methylation patterns of the plasmids before and after transfection and nuclear uptake using the restriction endonucleases Mbo I and Eco RII. Non-integrated plasmid DNAs were recovered from transfected cells by the method of Hirt (23). Analysis of the high molecular weight nuclear DNAs in these experiments gave no evidence of plasmid integrations in these series of experiments (results not shown).

Procedures have been reported for histone protein isolation and analysis on acid/urea containing polyacrylamide gels (14). Silver staining of proteins in polyacrylamide gels was by modification of the method of Merrill et al. (26). Micrococcal nuclease and DNase I were obtained from either Sigma Chemical Co. or Worthington Biochemical Co. and used as described.

RESULTS

Fate of Non-replicating Plasmid DNA Transfected by the CaPO₄ Method

Viral DNA molecules that transcribe and replicate in the nucleus, such as SV40 and polyoma (27) as well as papilloma (28), are known to be associated with cellular histones and nucleosomes in the form of minichromosome structures. Innis and Scott (22) have reported that a recombinant plasmid containing the SV40 viral origin region replicates as a minichromosome in the nucleus of COS-1 cells transfected by the DEAE-dextran procedure. Likewise, Cereghini and Yaniv, using similar techniques have recently demonstrated nuclear chromatin assembly on both replicating and non-replicating plasmids transfected into mammalian cells (29).

In contrast, little is known about the fate of plasmid DNAs introduced into mammalian cells by the widely used CaPO₄ method of transfection. The results shown in Figure 1 are, therefore, of interest since they demonstrate that, under appropriate conditions, non-replicating plasmid DNAs can be assembled into a typical chromatin structure by mammalian cells transfected by the CaPO₄ method. Panel A shows the results of an analysis of the DNA fragments released by a light micrococcal nuclease digestion of nuclei isolated from CV-1 cells 48 h after transfection with the plasmid pSV2cat. The DNA fragments released from both non-transfected control cells (lane 1), and from plasmid transfected cells (lane 2), were separated by electrophoresis in an agarose gel, transferred to a nitrocellulose filter and hybridized to a radioactive probe for the pSV2cat plasmid DNA. Lanes 1 and 2 show the ethidium bromide stained DNA fragments prior to transfer and lanes 3 (control) and 4 (transfected) the autoradiographs of these fragments after hybridization. The autoradiograph clearly demonstrates that at least some of the transfected pSV2cat DNA is assembled into a chromatin structure similar to that of the host cell nucleus with a periodic nucleosome repeat of about 190 bp.

Panel B of Fig. 1 show the result of an even more stringent test for the de

Figure 1. Assembly of CaPO_4 transfected plasmid DNA into nucleosomal chromatin.

PANEL A: Forty-eight hours after transfection of CV-1 cells with pSV2cat DNA ($10 \mu\text{g}/3 \times 10^5$ cells), nuclear nucleosome fragments released by brief micrococcal nuclease digestion (0.65 units/O.D.₂₆₀ units; 22°C , 4 min) were isolated and analyzed (lanes 2 and 4), along with fragments from matched mock-transfected (control) cells (lanes 1 and 3) by electrophoretic separation on a 1.4% agarose gel followed by Southern transfer to a nitrocellulose filter and hybridization to a nick translated (^{32}P)-labeled pSV2cat (spec. act. 5×10^8 dpm/ μg) probe. Lanes 1 and 2, photograph of ethidium bromide stained gel prior to transfer with "m", monomer, "d", dimer and "t", trimer nucleosome sizes of DNA indicated. Lanes 3 and 4, autoradiograph of DNA fragments shown in lanes 1 and 2.

PANEL B: Twenty-four hours after transfection of NIH/3T3 cells with pSV2gpt (at $10 \mu\text{g}$ of plasmid per 3×10^5 cells) nuclei were isolated from eight 10 cm tissue culture dishes, digested with micrococcal nuclease (as in panel A), and the released chromatin fragments separated by centrifugation on a 10-30% sucrose density gradient. Fractions were collected from the bottom of the tube and the optical density scanned (solid line, A_{260}). The fractions corresponding to the 11S monomer nucleosome particles were pooled and the DNA isolated. The monomer DNA was electrophoresed on a 1.4% agarose gel (lane 2), along with "marker" nucleosome DNA fragments isolated by micrococcal nuclease digestion from mock-transfected, control, NIH/3T3 cells (lane 1). Lanes 3 (control) and 4 (monomer nucleosome) show the autoradiograph of DNA fragments shown in the ethidium bromide stained gel (lanes 1 and 2) after transfer to a nitrocellulose filter and hybridized to nick translated (^{32}P)-labeled pSV2gpt (spec. act. $2.3 \times 10^8/\mu\text{g}$) probe. Labels m, d, and t as in panel A.

novo chromatin assembly of transfected plasmid DNAs. Twenty-four hours after transfection of NIH/3T3 cells with pSV2gpt, nuclei were isolated and lightly digested with micrococcal nuclease. The released chromatin fragments were

Figure 2. Isolation of non-replicating plasmid minichromosomes from CaPO_4 transfected cells. Seventy-two hours after transfection of CV-1 cells with excess pSV2neo plasmid DNA ($30 \mu\text{g}/3 \times 10^5$ cells), the cells were harvested. Nuclei were isolated and minichromosome nucleoprotein complexes isolated by centrifugation on a 5-20% sucrose gradient as described in Methods. DNA from the gradient fractions was collected and electrophoresed on 1.2% agarose gels with marker pSV2neo plasmid DNAs flanking the ends of the lanes containing the gradient fractions. The separated DNAs were transferred to nitrocellulose filters and hybridized to (^{32}P) -pSV2neo probes and the resulting autoradiograph shown here. Fractions 6-8 contain the Form I, supercoiled, plasmid DNAs isolated as minichromosomes which have a sedimentation value of approximately 70S. Labels: I, II, and III indicate supercoiled, nick-circular, and linear forms of the pSV2neo plasmid DNAs, respectively.

separated by centrifugation on a linear sucrose gradient and the fractions corresponding to 11S monomer nucleosome particles collected and the DNA isolated. Samples of the isolated monomer DNA were electrophoresed on an agarose gel (Fig. 1B, lane 2) along with marker DNA samples from lightly micrococcal nuclease digested nuclei of non-transfected NIH/3T3 cells (Fig. 1B, lane 1). The electrophoretically separated DNA fragments were transferred to a nitrocellulose filter and hybridized to a radioactive probe for the pSV2gpt plasmid DNA. The autoradiograph of the hybridized blot (Fig. 1B, lanes 3 and 4) clearly shows that the monomer nucleosome DNA isolated from transfected cells (lane 4), but not that isolated from non-transfected control cells (lane 3) contains sequences homologous to the newly introduced plasmid.

Minichromosome Isolation from CaPO_4 Transfected Cells

The results shown in Fig. 2 demonstrate that the chromatin formed on

plasmids introduced by the CaPO_4 method can be recovered from the transfected cells as minichromosomes. Seventy-two hours after CV-1 cells were transfected with an excess (see below) of pSV2neo plasmid DNA ($30 \mu\text{g}/3 \times 10^5$ cells) minichromosome complexes were extracted from isolated nuclei and purified by centrifugation on a sucrose density gradient. Fig. 2 (lanes 6-8) demonstrates that nucleoprotein complexes containing Form I supercoiled plasmid DNA were recovered which sediment in the sucrose gradient in a manner similar to the 70S minichromosome structures of SV40 chromatin (cf. 27). However, also evident in the figure is the fact that an appreciable amount (in this case >90%) of the transfected nuclear plasmid DNA sediments to the bottom of the gradient in an apparently aggregated or precipitated form.

Efficiency of Chromatin Assembly in CaPO_4 Transfected Cells

Often non-nucleosomal forms of CaPO_4 transfected plasmid DNA (which appear as a background "smear" of hybridizable plasmid material between adjacent nucleosomal DNA bands on the autoradiographs of gels) can be observed in the micrococcal nuclease digests of nuclei from recipient cells (results not shown). With increasing concentrations of transfected DNA above an optimal amount (determined to be approximately $5\text{--}10 \mu\text{g}/3 \times 10^5$ cells) the amount of this hybridizable "smear" greatly increases until a point is sometimes reached at which the superimposed plasmid nucleosome-repeat ladder is obscured (data not shown). The reasons for, or causes of, this hybridizable "background" of non-nucleosomal plasmid material are unknown.

Regardless of its origin, the existence of non-nucleosomal forms of transfected DNAs (in extreme cases such as that shown in Fig. 2, sometimes representing greater than 80-90% of the nuclear plasmid material) makes precise quantitative estimates of the amount of CaPO_4 transfected plasmid DNA actually assembled into chromatin structures difficult and subject to considerable uncertainty. Nevertheless, experiments were performed to try and assess the relative efficiency of chromatin assembly in recipient cells using for transfections supercoiled plasmid DNAs previously labeled in vivo to high specific activities with radioactive thymidine.

The results of these experiments indicated that under optimal conditions for potential chromatin assembly of calcium phosphate transfected DNA (see above) only approximately 60-70% of the plasmids reaching the nucleus within 24 h of transfection (approximately 0.02-0.03% of the radioactive DNA initially supplied to the cells) are assembled into chromatin (data not shown). In making these estimates, sucrose gradient centrifugation (cf., Fig. 2) was used to separate minichromosomal from non-nucleosomal forms of transfected radioactive plasmid

TABLE I

Saturation of the template capacity of plasmid pRSVcat transfected into CV-1 cells by the CaPO_4 method.

Concentration of Transfecting DNA	% Acetylated CM*		Relative Increase
	(-) Butyrate	(+) Butyrate	
1 μg	5	76	16x
3 μg	58	97	1.7x
5 μg	70	98	1.4x
10 μg	95	98	1.0x

*Radioactive spots on the thin-layer chromatography plate corresponding to the various acetylated forms of (^{14}C)-chloramphenicol (CM) were isolated and counted in a scintillation counter. The percentage of acetylated CM included both monoacetylated (two species) and diacetylated forms (where present) of chloramphenicol in each reaction mixture.

DNAs. Based on these calculations, and on the likelihood that only about 5% of the cells within a recipient population actually accumulate transfected DNA within their nuclei (30), under optimal conditions each "active" nucleus would be expected to contain about $6\text{--}7 \times 10^3$ plasmid molecules assembled into minichromosome structures.

Butyrate Effects on Transient CAT Gene Expression

Table I shows the effects of butyrate treatment on CAT gene expression in CV-1 cells transfected with increasing amounts of pRSVcat (1, 3, 5 and 10 μg) quantitatively expressed in terms of the percentage of chloramphenicol converted to acetylated forms by extracts of the transfected cells. The results clearly demonstrate that butyrate treatment markedly stimulates CAT gene expression when cells are transfected with low concentrations of plasmid DNA (e.g., 1 μg) but this relative stimulatory effect decreases with increasing concentrations of transfected DNA. Furthermore, it is evident that under normal transfection conditions (i.e., without butyrate treatment) with increasing concentrations of transfected plasmid DNA (1-10 μg) the transcription/translation apparatus of the recipient cells appears to become saturated at around 10 μg of transfected DNA. However, with butyrate treatment apparent saturation of the recipient cell transcription/translation apparatus occurs at much lower concentrations (1-3 μg) of transfected DNA suggesting either that butyrate treatment somehow increases the efficiency of transcription of transfected plasmid templates or that it somehow increases the efficiency of the cellular translation apparatus.

A direct effect (of the magnitude observed) on the translational apparatus itself seemed to us to be unlikely since normally the short-term butyrate treatment administered to the recipient cells occurs 24-48 h prior to the actual performance of the CAT enzyme assays. We, therefore, investigated the possibility

that butyrate treatment might alter the structure of the chromatin assembled on the plasmids soon after transfection.

Butyrate Effects on Plasmid Chromatin Structure

Fig. 3 shows the results of initial experiments designed to test the effects of butyrate treatment on transfected plasmid chromatin structure. Panel A of the figure demonstrates that butyrate treatment stimulates CAT enzyme activity by about 14X when CV-1 cells are transfected with pSV2cat (5 μ g) and assayed 36 h later. Panel B shows that short-term butyrate treatment (10 mM) for 12 h immediately following transfection also, as expected, induces histone hyperacetylation in the host cell chromatin.

With these findings, it seemed appropriate to investigate whether the newly assembled plasmid minichromosomes formed in the butyrate-treated cells might likewise contain hyperacetylated histones. However, owing to numerous technical difficulties, we were initially unable to recover sufficient plasmid minichromosome material for direct histone protein analysis. We, therefore, assayed for the possible presence of such highly modified histones on the plasmid minichromosomes by an indirect method utilizing the fact that chromatin containing hyperacetylated histones is considerably more sensitive to DNase I digestion than is chromatin with low or normal levels of acetylation.

Fig. 3C shows the results of two different nuclease digestion experiments. In the first experiment, nuclei from control (untreated) CV-1 cells transfected with (14 C)-thymidine labeled pSV2cat plasmid (5 μ g) were combined with nuclei from an equal number of butyrate-treated (experimental) CV-1 cells transfected with equivalent amounts of (3 H)-thymidine labeled plasmid (5 μ g) DNA and the mixture digested with DNase I. The acid-insoluble (10% TCA, 4°C) radioactivity of each of the two isotopes remaining in the nuclear preparation was determined at different time points during digestion. From the graph shown in Fig. 3C, it is evident that initially (within the first 4 min) the plasmid DNA from butyrate treated cells is digested at a rate that is 2-3 times faster than that of plasmid DNA from untreated cells. These qualitative results were confirmed by a second, similar but independent, line of experiments the results of which are shown in the inserted box of Fig. 3C. In this experiment, equal numbers of nuclei from either control (untreated) CV-1 cells (BA-) or butyrate treated CV-1 cells (BA+) were digested with DNase I for the times indicated and the nuclease-resistant DNAs isolated from each of the preparations and spotted (at equal concentrations) on a nitrocellulose filter. This DNA dot-blot was hybridized to nick translated pSV2cat plasmid DNA and the resulting autoradiograph shown in the insert of Fig. 3C. From an analysis of this autoradiograph it is again apparent that plasmid DNA from

Figure 3. Butyrate effects on non-replicating plasmid chromatin structure.

PANEL A: Assay of CAT enzyme activity in CV-1 cells transfected with pSV2cat DNA (10 μ g) by the CaPO_4 method with or without subsequent short-term butyrate treatment (10 mM, 12 h). Autoradiograph of a thin layer chromatograph on which various forms of (^{14}C)-chloramphenicol (un = unacetylated; mono = monoacetylated, two species) found in cell extracts have been separated. Lane 1, transfected control cells without butyrate treatment; lane 2, transfected cells with butyrate treatment; lane 3, reference standard (^{14}C)-chloroamphenicol (unacetylated). Ori is the origin spot of the thin layer plate.

PANEL B: Acid/urea polyacrylamide gel (15%) electrophoretic separation patterns of the histone proteins (H1, H2a, H2b, H3 and H4) isolated from either transfected control cells without butyrate (lane 1) or transfected cells exposed to butyrate (10 mM) for 12 h and then immediately harvested (lane 2). Acetylated forms of the H4

and H3 histone proteins are indicated as A₀, A₁, A₂, A₃ indicating 0, 1, 2 or 3 secondary, internal, post-synthetic acetylations of the proteins.

PANEL C: DNase I digestion sensitivity of transfected pSV2cat DNA in CV-1 cells with (○ - ○), or without (● - ●) butyrate treatment. Twenty-four hours after transfection of CV-1 cells with either (³H)-pSV2cat (butyrate treated cells) or (¹⁴C)-pSV2cat (untreated control cells), nuclei were isolated and digested with DNase I (40 units/ml, 37° C) for the times indicated. Acid-insoluble radioactivity (10% TCA, 4° C) of each of the isotopes (with appropriate corrections) remaining in the nuclei was then determined. Key: ● - ●, (¹⁴C)-pSV2cat control cells (cpm x 10⁻² scale); ○ - ○, (³H)-pSV2cat transfected cells treated with butyrate (cpm x 10⁻³ scale).

INSERT: Autoradiograph of dot-blots of nuclear DNA isolated from CV-1 cells transfected with pSV2cat plasmid DNA with (+BA) or without (-BA) subsequent butyrate treatment. The nuclei were isolated 24 h after transfection, digested with DNase I (as above), DNA isolated and spotted at equal concentrations on a nitrocellulose filter and hybridized with a (³²P)-pSV2cat probe (spec. act. = 2.6 x 10⁸ dpm/μg).

the nuclei of butyrate treated cells digested considerably faster than the plasmid DNA in control cells.

Replicating Minichromosomes Isolated from DEAE-Dextran Transfected Cells

One possible interpretation of the results presented in Fig. 3 is that butyrate treatment actually prevents the assembly of naked plasmid DNA into minichromosomal forms of chromatin, thus accounting for its increased DNase I digestion sensitivity. Since in previous experiments we were unable to isolate sufficient minichromosome material for direct protein analysis from cells transfected by the CaPO₄ method with non-replicating plasmids, to further investigate this possibility we introduced plasmid pSV2cat into COS-1 cells (in which the plasmid can efficiently replicate; data not shown) by the DEAE-dextran transfection technique. Fig. 4 demonstrates that butyrate treatment of cells transfected by this method does not prevent the assembly of the plasmids into nucleosome-containing minichromosomes. Panel A of the figure shows that micrococcal nuclease digestion of the nuclei of butyrate treated cells, 24 h after transfection, produces a characteristic chromatin nucleosome repeat ladder. Panel B of the figure demonstrates that minichromosome structures can also be isolated by sucrose gradient centrifugation of nuclear extracts from butyrate treated cells 72 h after transfection. An important point to note in Panel B of Fig. 4 is the apparent lack of precipitated or aggregated plasmid material sedimenting to the bottom of the gradient. This finding suggests, in agreement with the results of others (29), that under appropriate conditions, essentially all of the plasmid DNA reaching the nuclei of DEAE-dextran transfected cells is assembled into chromatin.

Minichromosomes from Butyrate-Treated Cells Contain Hyperacetylated Histones

Fig. 5 shows results indicating that replicating pSV2cat minichromosomes isolated from butyrate treated COS-1 cells 4 days (96 h) after transfection by the

Figure 4. Minichromosomes isolated from DEAE-dextran transfected cells treated with butyrate. **PANEL A:** pSV2cat DNA was transfected into COS-1 cells ($5 \mu\text{g}/3 \times 10^5$ cells) by a modification of the DEAE-dextran method. Transfected cells were treated with butyrate (10 mM) for 12 h, the fatty acid removed, and the cells harvested after a further 12 h of incubation (24 h post-transfection). Isolated nuclei were digested with micrococcal nuclease and nucleosomal chromatin fragments separated and analyzed as in Fig. 2. Lanes 1 and 2, ethidium bromide stained DNA gels; lanes 3 and 4, autoradiograph of DNA shown in lanes 1 and 2 transferred to nitrocellulose and hybridized to a (^{32}P)-pSV2cat probe (spec. act. 1.2×10^8 cpm/ μg). Lanes 1 and 3, mock-transferred COS-1 cells treated with butyrate (controls); lanes 2 and 4, pSV2cat transfected COS-1 cells treated with butyrate. Labels as in Fig. 1. **PANEL B:** Minichromosomes isolated from pSV2cat transfected COS-1 treated with butyrate and harvested 72 h post-transfection. Techniques as in Fig. 2. Autoradiograph of Southern transferred fractions from a sucrose density centrifugation gradient hybridized to a (^{32}P)-pSV2cat probe (spec. act. 2.7×10^8 cpm/ μg). Labels as in Fig. 2.

Figure 5. pSV2cat Minichromosomes from butyrate-treated COS-1 cells contain hyperacetylated histones. Twenty-five 10 cm petri dishes, each containing 5×10^5 COS-1 cells, were individually transfected with pSV2cat DNA (5 μ g) by the DEAE-dextran method and then exposed for 12 h to sodium butyrate (10 mM). The butyrate-containing medium was then removed and the cells cultured for an additional 84 h in fresh medium without butyrate. At 96 h post-transfection, minichromosomes were isolated from the cell nuclei as described in Fig. 5 and the histones extracted with 0.4 N H_2SO_4 .

PANEL A: Acid/urea polyacrylamide gel (15%) electrophoretic separation of histones as described in (14) and stained by a modification of the silver method. Lane 1, histones (0.25 μ g) from transfected COS-1 cells prior to butyrate treatment; lane 2, histones (0.5 μ g) from transfected COS-1 cells 12 h after butyrate addition; lane 3, histone (0.25 μ g) isolated from butyrate-treated, transfected COS-1 cells at the time of harvesting (96 h post-transfection); lane 4, histones extracted from replicating pSV2cat minichromosomes 96 h after transfection. The slower relative mobility of these histone proteins compared to the standards in lanes 1-3 is due to the very low amount of protein present in this sample compared to the other lanes.

PANEL B: Optical density scans (A_{550}) of the silver-stained electrophoretically separated histone proteins shown in panel A. Labels are as in panel A and Fig. 4.

DEAE-dextran method contain highly acetylated forms of nucleosomal histones. Panel A shows a silver stained acid/urea polyacrylamide gel of electrophoretically separated total cellular histones from pSV2cat transfected COS-1 cells immediately after transfection but prior to butyrate treatment (lane 1), after 12 h of butyrate treatment following transfection (lane 2) and at the time of cell harvesting and minichromosome isolation 96 h after transfection (84 h after removal of the butyrate) (lane 3). Lane 4 shows the profile of acid-extracted histones isolated from sucrose gradient purified minichromosomes extracted from butyrate treated cells 96 h after transfection. Panel B shows optical density scans of the stained gel profiles shown in panel A of the figure. Three points of importance should be noted from this figure: 1) The isolated pSV2cat minichromosomes contain highly acetylated forms of histone H4 (lane 4) whereas the bulk histones of the host cell chromatin (lane 3) show little indication of histone hyperacetylation; 2) The relative amounts of the mono-, di-, tri- and tetra-acetylated forms of histone H4 found in the isolated minichromosomes are very different from those of these same acetylated forms of histone H4 found in the host cell chromatin immediately after the butyrate treatment following transfection. In the minichromosomes, the tri- and tetra-acetylated forms of histone H4 are in considerable excess over the less highly acetylated forms of histone (lane 4) whereas the reverse is true for the H4 histones of butyrate treated COS-1 cell chromatin 12 h after transfection (lane 2); 3) Histone H1 is considerably depleted or absent from the isolated minichromosomes (lane 4) whereas it is readily detected in all of the host cell chromatin extracts (lanes 1-3).

DISCUSSION

Evidence that exogenous DNA introduced by transfection or microinjection techniques into eukaryotic cells establishes a regular chromatin structure that is important for the control of gene expression has come from a number of sources. For example, it has been shown for the herpes thymidine kinase gene transfected into mouse L cells that once integrated into the cellular DNA, it assembled as chromatin with a normal nucleosomal repeat (5). Additional experiments have demonstrated that modulations in the chromatin structure of the thymidine kinase gene is correlated with the regulation of expression of this gene (33). Other workers have shown that non-integrated plasmids (both non-replicating and replicating) introduced into cells by DEAE-dextran transfection assemble into nucleosomal forms of chromatin (29) and replicating minichromosomes containing plasmid sequences have been isolated from the nuclei of transfected cells (22). However, perhaps the most direct demonstration of the importance of chromatin assembly in

the functioning of exogenously supplied genes comes from microinjection experiments in *Xenopus* oocytes. Mertz has shown that when purified, circular SV40 DNA is injected into the germinal vesicle, a necessary but not sufficient condition for the efficient transcription of the viral genes by endogenous RNA polymerase II is for the DNA to be assembled into a regularly spaced nucleosomal form of negatively supercoiled chromatin (34). Microinjected unit-length linear forms of SV40 DNA failed to reconstitute into such minichromosome structures and were several orders of magnitude less efficient in the transcription of viral genes than the injected circular forms (35).

Consistent with the suggestion of the importance of chromatin assembly on the expression of foreign DNAs (34), we have demonstrated that both non-replicating and replicating plasmids introduced into cells by either the CaPO_4 or DEAE-dextran transfection techniques can be assembled in the nucleus into minichromosome structures whose regular nucleosome spacings (approximately 190 bp) closely resembles that of the endogenous host cell chromatin. However a consistent difference in efficiency of plasmid chromatin assembly between the CaPO_4 and the DEAE-dextran methods of transfection was observed. Whereas essentially all of the exogenously supplied plasmid DNA (in the range of 1-5 $\mu\text{g}/3 \times 10^5$ cells) reaching the nucleus of cells transfected by the DEAE-dextran method was assembled into minichromosomes (Fig. 4 and unpublished observations), apparently not all of the CaPO_4 transfected DNA that reaches the host cell nucleus is necessarily assembled into regularly repeating nucleosomal structures but often appears as an aggregated or precipitated form of plasmid DNA (Fig. 2 and unpublished observations). The precise nature or cause of this non-nucleosomal form of DNA is unknown but may partially account for the observation that the CaPO_4 method of transfection is, for certain situations such as short-term transient expression assays, considerably less efficient than the DEAE-dextran method of transfection (16, 18). Perhaps for similar reasons, the CaPO_4 method seems to be superior as a method for selecting stable transformants for a given transfected gene (18).

Butyrate treatment of cells transfected with pSV2cat markedly changes the physical properties of the chromatin assembled on the plasmid DNA since it greatly increases its sensitivity (relative to untreated controls) to digestion by DNase I (Fig. 3C). Although not conclusive, this finding supports our previous speculation that butyrate treatment of cells might stimulate transient gene expression by facilitating *de novo* assembly of the transfected DNA into an "active" chromatin structure (7). Much stronger experimental support for this idea comes from the results in Fig. 5 which show that replicating plasmid minichromosomes isolated from

butyrate treated cells exhibit other features believed to be characteristic of an "active" chromatin structure, highly acetylated forms of histone H4 and depleted amounts of histone H1.

Implicit in the suggestion that butyrate might stimulate transient gene expression by facilitating the formation of an "active" chromatin structure on transfected plasmids is the assumption that most of the newly introduced DNA would not normally be so assembled. This idea is consistent with the findings of others that only a small fraction of the minichromosomes assembled on plasmid DNAs injected into Xenopus oocytes is transcriptionally active as evidenced by electron microscopic observations (4) and with the fact that two different populations of assembled minichromosomes, one active (or "dynamic") and the other inactive (or "static") can also be biochemically detected in this system (36). In this context, it seems reasonable to suggest that butyrate treatment shifts the relative proportion of such "dynamic" and "static" minichromosomes assembled on newly introduced plasmid molecules in transfected cells. The recent findings of Partington et al. (37) that butyrate treatment markedly stimulates the expression of the human gamma-globin gene microinjected into Xenopus oocytes may also be relevant in this regard.

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