Transcriptional Regulation of Mammalian Genes in Vivo

A TALE OF TWO TEMPLATES*

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The past two decades have brought a major evolution in our understanding of promoter structure, transcription factors, and mechanisms by which transcriptional initiation is regulated in eukaryotes. Multiple approaches have been used to establish current models for transcriptional regulation, including the development of in vitro transcription systems using either naked DNA or reconstituted chromatin, genetic analysis of gene regulation in yeast and Drosophila, and in vivo analysis of either endogenous cellular genes or transiently transfected, exogenous gene promoters. It is now clear that chromatin structure, once considered to be transparent to the process of transcription, plays an important role in the regulation of gene expression (reviewed in Ref. 1). Since all cellular genes are packaged into ordered chromatin structures, an understanding of the mechanisms by which nucleoprotein structure influences transcription activation is necessary for a complete paradigm of gene regulation in higher eukaryotes.

Studies on regulation of endogenous mammalian genes are challenging due to the lack of genetic techniques available in yeast and Drosophila systems, which allow targeted insertion of promoters and gene disruption. Structural analysis of integrated gene promoters in mammalian cells requires the time-consuming generation of multiple stable cell lines or pools, in which the integrated genes would be subject to position effects from surrounding chromatin. Therefore, the identification and characterization of factors involved in mammalian gene expression have been addressed primarily through the use of transient transcription assays. In this approach, exogenous plasmid DNA, usually promoter/reporter constructs and transcription factor expression vectors, is introduced into cultured cells and expressed transiently, without replication or integration into the cellular genome. A variety of transfection methods has been utilized, including calcium phosphate precipitation (2), DEAE-dextran (3), electroporation (4, 5), and liposome-mediated transfer (6). These studies have resulted in an abundance of information about promoter structure (i.e. identification of cis-acting elements), transcription factor structure and function, and the characterization of transcription factor interactions that are part of various cellular regulatory pathways. However, in light of the accumulating evidence indicating a role for chromatin structure in transcription, it is appropriate to question whether transiently transfected gene promoters are adequate models for transcriptional regulatory mechanisms active on endogenous genes in ordered, replicated chromatin.

Structural Studies on Transiently Transfected DNA

The first issue to consider for transient templates is whether these molecules acquire physiologically spaced nucleosomes when introduced into cultured, mammalian cells. Since assembly of nucleosomes on endogenous genes is coupled to DNA replication, it is questionable whether transfected plasmids, which rarely carry mammalian replication origins, are properly assembled. This issue has not been extensively studied, but four studies are revealing (7-10). Cereghini and Yaniv (8) followed the superhelical status of plasmids (transfected by DEAE-dextran) containing fragments of the SV40 genome with varying abilities to replicate. Initially, the transfected DNA was in a relaxed form, but 24 h after transfection it had been converted to supercoiled forms, indicative of nucleosome formation. In fact, analysis of micrococcal nuclease digests from transfected cell nuclei showed the presence of clear, although short, nucleosomal ladders, whether the transfected DNA had been replicated or not. Repeat lengths were calculated to be 190 ± 15 bp, which is close to that calculated for bulk cellular chromatin (187 ± 5 bp) (7). In contrast, Jeong and Stein (7) showed that transfected plasmid DNA generated anomalous repeat patterns after micrococcal nuclease digestion. Repeat lengths from their extended ladders were calculated by more quantitative methodology and found to be 199 ± 5 bp. In addition, the smallest repeat unit was 280 bp in length, too long to be a nucleosome monomer and too short to be a nucleosome dimer. This pattern was found to be reproducible regardless of the cell lines tested, the size of the plasmid transfected, or the form of the plasmid transfected (supercoiled versus linear). Unlike bulk cellular chromatin, the majority of transfected DNA was observed to be associated with insoluble nuclear material. Although the significance of this latter observation is unclear, increased repeat lengths would suggest that transfected DNA is generally more open and accessible than cellular chromatin (Fig. 1B).

Using plasmids transfected by calcium phosphate precipitation, Reeves et al. (9) showed that nucleosomal ladders could be generated, which appeared indistinguishable from those of bulk cellular chromatin. In addition, mononucleosome-sized particles containing plasmid DNA isolated from transfected cells by sucrose gradients after micrococcal nuclease digestion of transfected cell nuclei. However, nuclear plasmid DNA was found to exist in an aggregated form, which, under certain transfection conditions, comprised 80–90% of the total amount. Also using calcium phosphate precipitation, Archer and colleagues (10) failed to observe nucleosome repeat patterns on transiently transfected MMTV promoter constructs under conditions in which nucleosomal ladders were detectable on stable, replicated forms of the promoter. Most of the plasmid DNA was completely digested by micrococcal nuclease, even at the lowest concentrations used. However, a fraction of the nucleosomal DNA was in a form completely resistant to digestion, even at the highest concentrations used, possibly representing the aggregated forms of transfected DNA observed in the previous study. Importantly, the localized nuclease hypersensitive region induced by the glucocorticoid receptor (GR) at the MMTV promoter (discussed below) could not be detected on the transiently introduced promoter construct, indicating that the chromatin remodeling event associated with activation of the stable, replicated form of the promoter does not occur on these templates (Fig. 1A). Taken together, the available structural data suggest that nucleosomes are deposited onto non-replicating DNA in transfected cells, but the overall structure may be incompletely organized into the nucleosome arrays characteristic of replicated cellular chromatin.

A major problem in correlating structural features of transfected templates with function concerns the fraction of the DNA that is actually transcriptionally active. One way to circumvent this problem is to examine the binding of transcription factors to the transfected DNA with the use of a gain-of-signal assay, such as exonuclease footprinting (11). This assay will detect binding events that occur on a small fraction of templates, thus permitting an evalu-
tion of molecular interactions on the active fraction. Archer and colleagues (10) used this approach to examine binding of factors to the proximal promoter of the MMTV promoter in a transfected, non-replicating form versus a stable, replicated form (10). When the replicating form of the promoter is activated by GR, two factors, NF1 and Oct1, bind in a hormone-dependent fashion (Fig. 1A) (12–14). In contrast, these factors are constitutively bound to the transfected MMTV template, and the amount of their binding does not increase upon activation of GR (Fig. 1B) (10, 12). Hormone-dependent binding of the TFIID basal factor complex on transient templates was observed, indicating that the footprinting assay could detect actual factor-loading events on transcriptionally active templates (12). A reasonable interpretation of these findings is that transfected DNA does not acquire the repressed chromatin structure of stable, replicated templates, and this structure is required for exclusion of ubiquitous factors.

Functional Studies of Transiently Transfected versus Stable Replicating Templates

Evidence of critical structural differences between transiently transfected DNA and cellular chromatin derives from functional studies comparing transiently transfected promoters and their stable, replicating counterparts. In some cases the two templates manifest similar responses, but there are a growing number of reports showing significant differences in behavior. Four specific examples will be briefly mentioned.

1) The α1-antitrypsin gene is actively transcribed in hepatocytes, but its expression is extinguished upon fusion of cultured hepatocytes with fibroblasts. Transiently transfected promoter constructs respond primarily to the activator, HNF-1 (15, 16); expression of this activator is also extinguished upon fusion (17, 18). Ectopic expression of HNF-1 results in activation of the transient promoter template in fibroblasts and hepatocyte/fibroblast fusions (15). However, the endogenous α1-antitrypsin gene cannot be activated in either of these circumstances, and its fusion-dependent extinction cannot be prevented by constitutively expressed HNF-1 (15), indicating that the regulation of the endogenous promoter is more complex than indicated by studies with the transfected version.

2) Studies on the integrated HIV genome provide another example of functional differences between transfected and chromosomal templates. In the ACH-2 and U-1 cell lines, the HIV genome is integrated, but expression of viral RNA and levels of viral replication are low (19, 20). The activity of a transiently transfected HIV promoter construct is low in U-1 cells but is constitutively high in ACH-2 cells (20). The U-1 cells lack functional Tat protein, which is viral encoded and necessary for viral transcription and replication, because both the transiently transfected HIV promoter construct and the integrated viral genome were activated upon addition of exogenous Tat protein. However, in ACH-2 cells, RNA generated from the integrated genome could not be increased by addition of exogenous Tat protein, suggesting additional requirements are necessary for activation from a replicated chromatin environment (20). The integrated HIV genome in ACH-2 cells was not defective or irreversibly inactive, since treatment of the cells with the protein kinase C activator, phorbol 12-myristate 13-acetate, resulted in increased expression of viral RNA and viral replication (19, 20).

3) Regulation of the entire human β-globin gene genomic locus is controlled by a large region containing four erythroid-specific DNase I-hypersensitive sites (HS) (21, 22), referred to collectively as the locus control region. Examination of each hypersensitive region through transient transfection revealed that only the HS2 region had classical enhancer activity or the ability to activate a minimal promoter from a distance in an orientation-dependent fashion (23). In contrast, all four regions are required for full, copy number-dependent, position-independent expression in transgenic animals (24, 25). The HS2 region by itself is ineffective in allowing activation of single copy transgenes (25, 26). Only through examination of stable replicating forms of the β-globin gene was it determined that HS3 contains chromatin opening activity (25).

4) Activation of the c-jun promoter in F9 embryonal carcinoma cells by retinoic acid treatment or expression of the adenovirus early 1A (E1A) protein (necessary for adenovirus transcription and replication) presents another interesting example of functional differences between transiently transfected and integrated templates. A stable, integrated c-jun/chloramphenicol acetyltransferase construct is activated at least 50-fold under these conditions, whereas the same construct, when transiently transfected, is activated only 2–3-fold (27). Remarkably, the weak activation of the latter is dependent on a 12-O-tetradecanoylphorbol-13-acetate response element (28), but strong activation of the former is dependent on different elements, a series of direct and inverted repeats (27). Interestingly, some of these sequences are bound in a retinoic acid- and E1A-dependent fashion by a complex containing p300 (29), which has recently been found to be a histone acetyltransferase (30). These results suggest that a repressive chromatin structure at the c-jun promoter must be remodelled and the transient template does not require or cannot use this activity.

Each of these examples suggests that transiently transfected promoters, while they may form nucleosomal structures, are not subject to the same regulatory mechanisms which operate on their integrated counterparts if those particular mechanisms involve complex chromatin structure. The nucleosomal arrays deposited on the transfected, non-replicating DNA are apparently not as repressive as those formed by replication-dependent chromatin assembly on endogenous genes. The functional data indicate that transcribed
Identification of activities required for the transition, in addition to the receptor itself, and characterization of the molecular nature of the transition. Required remodeling cofactors may include nucleosome replication mechanisms of activation. This approach has been especially fruitful in terms of identifying chromatin-specific events at the replicating promoter, not only in the depression step but also in the activation step.

Three lines of investigation indicate a strong role for chromatin in regulation of MMTV. The first concerns the kinetics of activation. GR-induced transcription of the replicated template is temporary, peaking 1 h after addition of hormone and declining to near basal levels by 24 h, even in the continued presence of steroid (12, 44). This decrease in transcription correlates with a reformation of the repressed chromatin structure at the promoter (44). In contrast, the transient template is active as long as glucocorticoids are present and is not refractory to reactivation if the steroid is removed (12).

The two MMTV templates also differ dramatically in their response to progestins. Both GR and the progesterone receptor (PR) bind to the same hormone response elements in the MMTV promoter (45). However, when the PR is transiently expressed, it is a poor activator of the replicating template but efficiently activates the transactivated template (Fig. 1C) (44, 46). In contrast, whether transiently expressed, the GR is an efficient activator of both templates (47). The two receptors also differ in their ability to induce the chromatin remodeling event at the replicating template; transiently expressed PR is unable to induce this structural transition (47). Since the transient template does not require remodeling, the PR is able to induce its transcription efficiency. These results show that activation of the replicating template has additional requirements beyond those necessary for activation of the transient template. In addition, the GR and PR have different requirements for remodeling of ordered chromatin; this differential may provide a possible mechanism for achieving selective gene activation in vivo.

The most striking difference in function between the two MMTV templates occurs in response to cAMP signaling. Whereas the transient template is synergistically activated by both glucocorticoids and cAMP, the replicating template is significantly repressed by cAMP in a glucocorticoid-independent fashion (14). In this case, differences in the nucleoprotein status of the promoter result in entirely different modes of regulation. Interestingly, cAMP-induced repression does not inhibit the GR-induced remodeling event at nucleosome B but results from inhibition in binding of Oct1 and the TFIID basal factor complex to sequences just upstream of the A nucleosome region (Fig. 1D) (14). This observation strongly suggests that, in addition to the repression step, there are chromatin-sensitive events in the second step of the bimodal mechanism by which transcription is activated at the MMTV template in ordered chromatin. These experimental findings illustrate the usefulness of the two-template approach for the identification of path-ways by which chromatin participates in the regulation of transcription.

Template comparison assays have not been limited to the MMTV promoter. Gerber et al. (36) employed this method to explore differentiation-induced activation of endogenous muscle-specific genes by MyoD and myogenin. Whereas both proteins activate transiently transfected templates, MyoD was better able to activate genomic copies of muscle-specific target genes. In fact, expression of MyoD correlated well with loosening of chromatin structure within the promoters of these genes. The template comparison assay was also used to identify domains of MyoD, which are nec-
Summary

The study of mammalian gene expression through the use of transient transfection assays has greatly expanded our knowledge of transcriptional mechanisms. However, transfected promoter constructs do not always serve as appropriate “stand-ins” for endogenous genes, particularly in cases where chromatin remodeling may take place. The examples described above indicate that a level of caution is advised when studying regulation of various promoters and transcription factor function with the use of transient transfection assays. When possible, function of the corresponding endogenous target genes and assist in the elucidation of regulatory mechanisms defined on transiently transfected, nonendogenous promoters should be tested to assess the validity of transient transfection assays. When possible, function of the corresponding endogenous target genes and assist in the elucidation of mechanistic involvement in chromatin transitions. As in the MyoD experiments (36), these studies will also allow identification of functional domains in these factors, which are necessary for chromatin remodeling. Functional differences between transiently transfected and stable replicating templates need not be considered artifactual but rather can be exploited to identify and characterize regulatory mechanisms that involve chromatin components. Full understanding of gene expression in vivo will not be achieved until these mechanisms are understood in detail.

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