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学 位 論 文 題 名

Distinctive Effects of the Epstein-Barr Virus Family of Repeats on Viral Latent Gene Promoter Activity and B-lymphocyte Transformation

(EB ウイルス FR 反復配列のウイルス潜伏感染遺伝子発現プロモーター活性および B リンパ球トランスフォーメーションに及ぼす異なる影響)

学位論文内容の要旨

Background and Objectives

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that is associated with various lymphoid and epithelial cancers, such as Burkitt's lymphoma (BL), gastric carcinoma, nasopharyngeal carcinoma and lymphomas in immunosuppressed patients. EBV infects B-lymphocytes in vitro and transforms them into indefinitely growing cells, known as lymphoblastoid cell lines (LCLs). In the LCLs, EBV establishes latent infection, and little progeny viruses are produced. Only a limited numbers of viral latent proteins, most of them are essential for B-cell transformation, are expressed in the LCLs.

The EBV genomes are maintained as circular, double-stranded DNAs (designated as episomes) in latently infected cells. Episomal maintenance is mediated by the following two viral elements: a *cis*-acting sequence, *oriP*, and a gene encoding a *trans*-acting protein, EBV nuclear antigen-1 (EBNA-1). The *oriP* sequence consists of two functional elements, the dyad symmetry element (DS) and the family of repeats (FR).

The FR consists of multiple copies of a 30-bp repeat unit, each unit consisting of a 16-bp palindromic sequence that constitutes an EBNA-1 binding site. Multiple EBNA-1 molecules bind to the FR sequence and transactivate the viral latent gene promoter, BamHI C promoter (Cp), which drives the expression of viral transforming proteins, such as EBN-A2, EBN-A3s, and EBNA-1 itself. EBNA-1 binding to the FR sequence also mediates the nuclear retention of EBV episomes and *oriP*-containing miniplasmids.

The number of 30-bp repeat units within the EBV FR varies between strains. It has been reported that the FR of the EBV B95-8 strain, a prototype EBV, consists of 29 copies of repeats (FR29). Importantly, the FR length is strictly maintained when the virus is maintained in cells. By contrast, the FR sequence tends to be partially deleted when it was subcloned in *E. coli*-based plasmid vectors. For example, the FR of commonly-used *oriP* miniplasmids (for example, pCEP4) consists of only 20 copies of repeats (FR20), and 9 copies of the repeats are missing compared to that of the original EBV B95-8 strain from which it is derived.

We got interested in investigating whether the full length FR of the EBV B95-8 strain plays a role in specific biological functions that cannot be mediated by the deleted FR. Thus, we compared the full length FR (FR29) and the deleted FR (FR20) by focusing on their transcriptional enhancer activities as well as their abilities to support B-cell transformation.

Materials and Methods

The FR lengths of latently infected EBVs were verified by Southern blotting. A low-copy-number plasmid vector was used to subclone the EBV B95-8 strain DNA fragment spanning the full length FR (FR29), which consists of 29 copies of repeats. The deleted version of FR (FR20) was obtained from pCEP plasmid. The luciferase reporter constructs containing either the full length FR (FR29) or the deleted FR (FR20), located upstream of viral latent gene promoter Cp, were constructed. The reporter constructs were introduced into various EBV-positive (Akata, B95-8, and P3HR-1 cells) and EBV-negative (BJAB) cell lines to examine the enhancer activities of FR29 and FR20. The effect of FR lengths on the replication efficiencies of *oriP*

miniplasmids were examined by transient replication assay in the EBV-positive Raji cells. The bacterial artificial chromosome (BAC) clone containing the entire genome of the EBV (Akata strain) was genetically engineered in *E. coli* to obtain two recombinant EBV genomes, one having FR29 (FR29-BAC) and the other having FR20 (FR20-BAC). The recombinant EBVs were then produced by utilizing P3HR-1 cells as virus producing cells, and they were subjected to B-cell transformation assay to determine their transforming titers.

Results

Southern blotting experiment revealed that the FR sizes vary between EBV strains, and that the sizes of the FR sequences do not change during the life cycle of each EBV strain. When EBV-positive cells with endogenous EBNA-1 expression were used for the reporter assay, we found that both the full-length FR (FR29) and the deleted FR (FR20) enhanced the activity of viral latent gene promoter (Cp), but the reporter construct of FR29 exhibited attenuated transactivation compared to that of the FR20 reporter. In EBV-negative cells, exogenous expression of EBNA-1 protein transactivated both the FR29 and FR20 reporter constructs, but again the FR29 reporter construct exhibited attenuated transactivation, supporting the results obtained from EBV-positive cells. We further dissected the FR sequence into parts and found that the 9 copies of 30-bp repeats, located at the 3'-end of the full length FR, is responsible for attenuating the transactivation of viral latent gene promoter.

We then examined how the difference of FR sizes of the test plasmids affects their replication efficiency. Transient replication assay using the EBV-positive Raji cells revealed no significant difference between the replication efficiency of FR29 test plasmid and that of FR20 test plasmid.

We then investigated the importance of the full length FR in the context of the EBV genome by using a recombinant EBV technology. The recombinant viruses of FR20-BAC and FR29-BAC were produced, and they were subjected to B-cell transformation assay. Both viruses infected B-lymphocytes and transformed them into LCLs. The results revealed that the recombinant virus of FR29-BAC exhibited significantly higher B-cell transformation efficiency compared to that of FR20-BAC.

Discussion

The FR sequence is a highly repetitive sequence with more than 20 copies of EBNA-1 binding sites, and EBNA-1 binding to the FR sequence is well known to enhance viral latent gene promoter, Cp. We found that the inclusion of the full length FR (FR29) in a reporter construct did not result in enhanced transcriptional regulation compared to the FR20 reporter construct. Therefore, it becomes apparent that the increased copies of EBNA-1-binding sites do not necessarily result in stronger enhancer activity.

Our results suggest that the 9 copies of 30-bp repeats (252-bp sequence, which is subject to deletion in *E. coli*-based plasmids) somehow contribute to the attenuated enhancer activity. The deleted 252-bp sequence contains a 128-bp complete dyad symmetry, which is expected to form a hairpin-like secondary structure. Interestingly, although primary sequences and the sizes of the FR are heterogeneous among various EBV strains, they all share a stretch of nucleotide sequence which can form similar secondary structures. Thus, the overall data support the idea that a putative hairpin-like structure plays a role in regulating the enhancer activity of the viral latent gene promoter.

The enhanced B-cell transformation ability obtained by the recombinant EBV having the full length FR highlights the importance of preserving the full length FR for the biological activities of EBV. There are at least two explanations for the enhanced transformation efficiency. First, the full length FR (FR29) can accommodate approximately 40% more EBNA1 molecules than the deleted FR, which results in the better maintenance of EBV episomes in the infected B-lymphocytes. Second, as the excess expression of viral transforming protein is known to be disadvantageous for B-cell transformation, preserving the full length FR is required for the appropriate expression levels of viral transforming proteins, which is likely to be critical for efficient B-cell transformation.

Conclusion

This study has clearly demonstrated that the integrity of repetitive sequences is sometimes necessary for viruses to function efficiently. We conclude that the presence of the full-length FR contributes to the precise regulation of the viral latent promoter and increases the efficiency of B-cell transformation.

学位論文審査の要旨

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Distinctive Effects of the Epstein-Barr Virus Family of Repeats on Viral Latent Gene Promoter Activity and B-lymphocyte Transformation

(EBウイルス FR反復配列のウイルス潜伏感染遺伝子発現プロモーター活性およびBリンパ球トランスフォーメーションに及ぼす異なる影響)

申請者は、まずEBウイルスゲノム（約170キロベースの2本鎖DNAゲノム）上の反復塩基配列の一つであるFamily of repeats配列(FR配列)について解説した。次にデータを示しつつ、FR配列は感染細胞内のEBウイルスゲノムではきわめて安定に維持されるにもかかわらず、大腸菌のプラスミドベクターにクローン化すると、頻繁に部分欠落することを示した。そして本研究の目的が、EBウイルスゲノム上において完全長FR配列が維持されていることの意義の解明であることを説明した。ついでB95-8株EBウイルスゲノムのFR配列(29コピー)をプラスミドベクターに初めて完全長のままクローン化したこと、リポーターアッセイにおいて、完全長FR配列は、部分欠落を有するFR配列(20コピー)に比べて、むしろ弱いエンハンサー活性を示すことを説明した。さらに完全長FR、ないし部分欠落FRを持つEBウイルスゲノムのBAC(bacterial artificial chromosome)を用いて組換えEBウイルスを産生し、トランスフォーム活性の差を見たところ、完全長FRを保持したBACクローン由来の組換えウイルスが、10倍近く効率よくBリンパ球を不死化できることを説明した。以上より、完全長FR配列を保持することは、EBウイルスがその生物学的活性を発揮する上で、重要な貢献をしていると結論した。

質疑応答として、まず有川教授より、大腸菌のベクターで部分欠落する9コピーのFR反復配列の意義について質問があった。それに対して申請者は、配列中に含まれる128塩基対のパリンドローム配列が形成する二次構造の重要性が考えられること、またFR配列内のこうした二次構造を形成しうる塩基配列は、多くのEBウイルス株において保存されていることを説明した。またFR配列を介したEBウイルスエピゾームの宿主染色体付着メカニズムについて、これを阻害することがEBウイルス関連疾患の治療に応用できるかという質問

があったが、これに対して EBNA1 蛋白質の機能阻害は治療への応用の可能性があることを説明した。

次いで志田教授より、本研究結果の普遍性に関して、別の EB ウイルス株である Akata 株の FR 配列の解析の有無について質問があったが、申請者は、Akata 株の FR 配列は低コピープラスミドを用いてもクローン化できなかったこと、その原因は不明であることを説明した。また EB ウイルス DNA をトランスフェクションして B リンパ球をトランスフォーム可能であるかという質問があった。申請者は、トランスフォーメーションの過程ではウイルス遺伝子発現プロモーターの切り替えを含むウイルス遺伝子の段階的発現が起こることが重要であることを説明した。この点に関して、高田教授から、特殊な実験系ではウイルス DNA のトランスフェクションだけでトランスフォームできるとのコメントがあった。

最後に高田教授より、FR 配列のコピー数の違いによるエンハンサー活性の違いが、潜伏感染遺伝子産物 EBNA1 量の違いに現れたかという点について質問があった。これに対して、今回用いた P3HR-1 株ウイルスとの混合ウイルスを用いた実験系では、B リンパ球感染後の EBNA1 蛋白質の発現経過を解析することが困難であり、EBNA1 の発現レベルへの影響は未解析であることを説明した。また FR 配列の違いが EB ウイルスミニプラスミドの核内維持効率に影響するかという質問に対しては、大きな影響を認めなかったことを説明した。

本学位論文は、EB ウイルスゲノム中の反復配列のコピー数を安定に保持することで、ウイルスが効率のよい B リンパ球トランスフォーム能を発揮していることを初めて明らかにしたものである。FR 配列の大腸菌のクローニングベクターにおける不安定性という一見些細な観察から、ウイルスの生物学的活性への影響を見出した本研究成果は高く評価できる。本研究で得られた知見は、リバースジェネティクス法による組換え EB ウイルス産生法の改善にもつながるもので、EB ウイルス学のさらなる進展に資するものと期待される。

審査員一同は、これらの成果を高く評価し、大学院過程における研鑽や取得単位なども併せ、申請者が博士（医学）の学位を受けるのに充分な資格を有するものと判定した。