
Heparin inhibits EcoRI endonuclease cleavage of DNA at certain EcoRI sites

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ABSTRACT

Studies presented here demonstrate that heparin inhibits EcoRI endonuclease cleavage of DNA whereas related proteoglycans show no effect. The inhibition occurs at particular EcoRI sites that are near or overlap with palindromic sequences in the murine λ_5 and Lyt-2 genes. Endogenous heparin from peritoneal mast cells co-isolates with DNA and inhibits digestion of peritoneal cell DNA at the inhibitable sites. Digestion of spleen DNA is inhibited at the same sites when commercial heparin is added prior to digestion. In both cases, the inhibition is abolished by pre-treating the DNA with heparinase. Thus, potential artifacts in restriction fragment length analyses could occur with DNA isolated either from cells that are naturally rich in heparin or from cells to which heparin has been added, e.g., as an anticoagulant.

INTRODUCTION

Heparin is a proteoglycan composed of sulfated polysaccharides attached to a core protein (1-3). Commercial heparin, which mainly contains the sulfated polysaccharide chains without the core protein, binds to a variety of positively charged molecules, including endonucleases, and is often used for chromatographic purification of EcoRI endonuclease and other proteins (4-7). It is also used routinely as an anticoagulant when isolating cells from blood.

Heparin is widely distributed in animal organs and is particularly abundant in mouse peritoneal mast cells (3, 9-11). In its natural form, it is similar to DNA in that it is a heterogeneous mixture of highly negatively charged macromolecules that are resistant to proteinase treatment. Thus, it often co-isolates with DNA and tends to contaminate DNA preparations from sources rich in heparin-containing cells such as the mouse peritoneum (3, 9, 12). In addition, it tends to contaminate DNA isolated from cells that have been harvested from blood to which heparin has been added as an anticoagulant (even though commercial heparin molecules are smaller in size) (8).

Studies presented here underscore the importance of such contamination by showing that the restriction endonuclease digestion of DNA at certain sites is inhibited by heparin. Southern analyses of EcoRI digests of spleen DNA using λ_5 genomic or

Lyt-2 cDNA probes yield bands whose sizes are predicted by the restriction sites in genomic maps. In contrast, Southern analyses of peritoneal DNA digests using the same probes yield additional bands that are larger than those predicted by the genomic maps. These larger bands are due to sufficient amounts of heparin co-purifying with DNA and heparin inhibiting EcoRI cleavage of DNA at EcoRI sites that are near or overlap with palindromic sequences. Thus, heparin co-purified with DNA may introduce artifacts in restriction fragment length analyses. Nevertheless, addition of appropriate amounts of heparin into EcoRI digestion mixture can produce controlled, partial digests of DNA that contains inhibitable sites.

MATERIALS AND METHODS

Materials

Restriction endonucleases were from New England Biolabs (Beverly, MA), United States Biochemical (Cleveland, OH) and Boehringer Mannheim Biochemicals (Indianapolis, IN). Proteinase K, RNase A and DNase I were from Boehringer Mannheim Biochemicals. Heparinase was from Sigma (St. Louis, MO). Heparin was from Upjohn (Kalamazoo, MI) and was precipitated in 0.3 M sodium acetate by 70% ethanol three times before use. Heparan sulfate, chondroitin-4-sulfate and chondroitin-6-sulfate were gifts from Dr. Merton R. Bernfield. λ_5 DNA probe was from Drs. Mark M. Davis and Fritz Melchers and the P₁-450 DNA probe was from Dr. James Whitlock.

Isolation of genomic DNA

Genomic DNA was isolated as described (13). Briefly, peritoneal cells and red blood cell lysed spleen cells were washed three times in TEN (20 mM Tris·Cl, pH 7.5, 2 mM EDTA and 150 mM NaCl), and resuspended in TEN at a concentration of about 5×10^7 cells/ml. An equal volume of TEN containing 0.4 mg/ml proteinase K and 0.4% SDS was added, mixed thoroughly and incubated at 37°C overnight. The mixture was extracted twice with phenol, three times with phenol/chloroform/isoamyl alcohol (25/24/1) and twice with chloroform/isoamyl alcohol (24/1). DNA was precipitated by ethanol, washed once in 70% ethanol, air dried and resuspended in TE (10 mM Tris·Cl, pH 7.5 and 0.1 mM EDTA) at 0.2 to 0.5 mg/ml.

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DNA digestion and Southern hybridization

5 μg genomic DNA was digested with EcoR I restriction endonuclease at 37°C in 30 μl of high salt buffer for about four hours. After another aliquot of enzyme was added, the mixture was incubated overnight. All digestions were done with excess restriction endonucleases (about 5 units enzyme per μg DNA). DNA was fractionated on 0.7% agarose gel and transferred to nitrocellulose filters. The filters were hybridized with appropriate probes labelled by random priming and followed by autoradiography. Same filters were re-hybridized with other probes following removal of hybridized probe by boiling in distilled water for 5 min.

RESULTS

Heparin inhibits EcoR I endonuclease digestion of DNA at a particular EcoR I site

Murine spleen DNA digested by EcoR I and probed with a λ_5 exon III-specific probe yields a single 1.4 kb band that is the expected size from complete digestion (Fig. 1A, lane 1) (14). However, spleen DNA digested in the presence of sufficient amount of heparin (10 to 0.08 μg heparin per 5 μg DNA) yields two bands, one at the expected 1.4 kb and another at 1.8 kb (Fig. 1A, lanes 2 to 5).

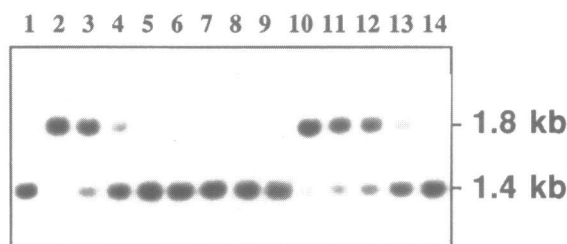
The appearance of the 1.8 kb fragment is not due to incomplete DNA digestion at all EcoR I sites. First, removal of the λ_5 probe and re-hybridization with other probes, including P₁-450 (Fig. 3A) (15), C λ 1, Ly-1, DHFR or Ii (data not shown), gives rise to discrete bands of the sizes expected from complete

digestion. Secondly, DNA digestion in the presence of heparin results in two discrete λ_5 hybridizing bands rather than the smear that would be expected if heparin inhibits EcoR I digestion at other nearby sites. Finally, the intensity of the 1.8 kb band is positively correlated with the amount of heparin added until saturation levels are reached. Further addition of heparin does not generate any new bands (Fig. 1A, lanes 2 to 5).

DNA digestion by EcoR I is not inhibited by other proteoglycans that are structurally related to heparin. Digestion of spleen DNA in the presence of heparan sulfate and hybridization with λ_5 probe yields only the 1.4 kb fragment (Fig. 1A, lanes 6 to 9). Similarly, digestion of spleen DNA in the presence of chondroitin-4-sulfate or chondroitin-6-sulfate and hybridization with λ_5 probe gives rise to only the 1.4 kb fragment (data not shown). Moreover, heparinase treatment of the mixture of spleen DNA and heparin before EcoR I digestion yields only the 1.4 kb fragment (Fig. 2A). Thus, heparin, but not other related proteoglycans, inhibits EcoR I endonuclease cleavage of DNA at a particular EcoR I site in the λ_5 gene.

Based on the λ_5 genomic map, the EcoR I site inhibited is the one in exon III (Fig. 1B) (14). When this site is blocked, a 1.8 kb fragment instead of the expected 1.4 kb fragment is generated.

A. Southern blot:



B. λ_5 genomic map:

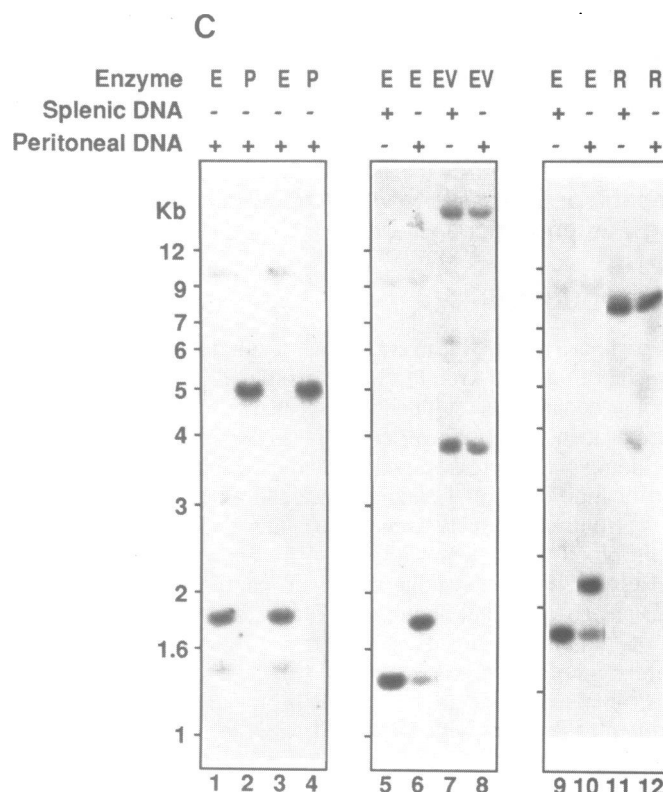


Figure 1. Heparin inhibits EcoR I endonuclease digestion of DNA at a particular EcoR I site. A, Southern blot. DNA was isolated from spleen and peritoneal cells, digested by EcoR I, fractionated on 0.7% agarose gel and hybridized with λ_5 DNA probe. Lane 1, digest of 5 μg spleen DNA; lanes 2 to 5, digests of 5 μg spleen DNA in the presence of 10, 2, 0.4 and 0.08 μg heparin, respectively; lanes 6 to 9, digests of 5 μg spleen DNA in the presence of 10, 2, 0.4 and 0.08 μg heparan sulfate, respectively; lane 10, digest of 5 μg peritoneal DNA; and lanes 11 to 14, digests of 4, 5, 5, and 5 mg spleen DNA in the presence of 1, 0.2, 0.04 and 0.008 μg peritoneal DNA, respectively. B, λ_5 genomic map (14). The 260 bp EcoR I-Xho II DNA fragment is used as hybridization probe. The 1.4 kb fragment is the expected λ_5 band from complete EcoR I digestion. The 1.8 kb fragment is generated when the EcoR I site in exon III is blocked. P, E, and EV represent Pst I, EcoR I and EcoR V sites, respectively. Exons are indicated by bold bars and numbered from I to III. C, heparin does not inhibit DNA digestion by Pst I, EcoR V or Rsa I. 5 μg spleen DNA or peritoneal DNA was digested by EcoR I (E), Pst I (P), EcoR V (EV) or Rsa I (R). DNA digests were fractionated and hybridized with λ_5 DNA probe as in Fig. 1A. Pst I digests give rise to the expected 5 kb fragment. EcoR V cleaves in the middle of the hybridization probe and thus its digests give rise to two hybridizing bands. Since the second Rsa I site and the second and the third EcoR V sites are not defined in the known DNA sequences, the fragment sizes of Rsa I and EcoR V digests can not be predicted. However, both spleen DNA and peritoneal DNA digests give rise to identical band pattern, suggesting that the digestion is not inhibited by heparin as determined with λ_5 probe. The weak bands at about 9 kb are generated from cross-hybridization to the immunoglobulin C λ 1 sequence.

Endogenous heparin in DNA isolated from peritoneal cells also inhibits EcoR I digestion at the EcoR I site in the λ_5 exon III

Mouse peritoneal cells are rich in heparin-containing mast cells (10). Since heparin co-purifies with DNA and is effective at inhibiting EcoR I digestion at relatively low concentrations (0.4 μg heparin per 5 μg DNA), it is not surprising that peritoneal DNA digested by EcoR I and hybridized with λ_5 yields both the 1.4 kb and the 1.8 kb bands (Fig. 1A, lane 10). In fact, the 1.8 kb band consistently predominates in peritoneal DNA digests, regardless of the age or sex of the peritoneal cell donors (BALB/c, C57BL/6 and CBA/Ca). In contrast, the 1.8 kb band is never seen with spleen DNA from any of these mice unless heparin is added to the digestion mixture.

The generation of the 1.8 kb fragment in EcoR I digestion of peritoneal DNA is clearly due to the presence of endogenously-derived heparin. Treating peritoneal DNA with heparinase before

digestion yields only the 1.4 kb fragment whereas treating this DNA with RNase A or proteinase K yields both the 1.4 kb and the 1.8 kb fragments (Fig. 2A and B). DNase I treatment degrades all DNA, however, the inhibitory activity remains (Fig. 2B). Furthermore, heparin co-purified with peritoneal DNA behaves identically to exogenous heparin. It inhibits EcoR I digestion of DNA at the EcoR I site in the λ_5 exon III but not at other nearby sites. Nevertheless, the co-purified heparin neither grossly alters λ_5 DNA nor is simply bound to DNA at the EcoR I site whose digestion it blocks, since digestion of peritoneal DNA by EcoR V or Rsa I is not inhibited (Fig. 1C). These EcoR V and Rsa I sites are in the λ_5 exon III, and the latter is only seven nucleotides away from the inhibited EcoR I site (Fig. 1 and 4).

The amount of heparin that co-purifies with peritoneal DNA is greater than the amount necessary to block digestion of this DNA. The co-purified heparin also blocks digestion of spleen DNA that is added to the peritoneal DNA prior to digestion. As indicated above, digestion of peritoneal DNA mainly yields the

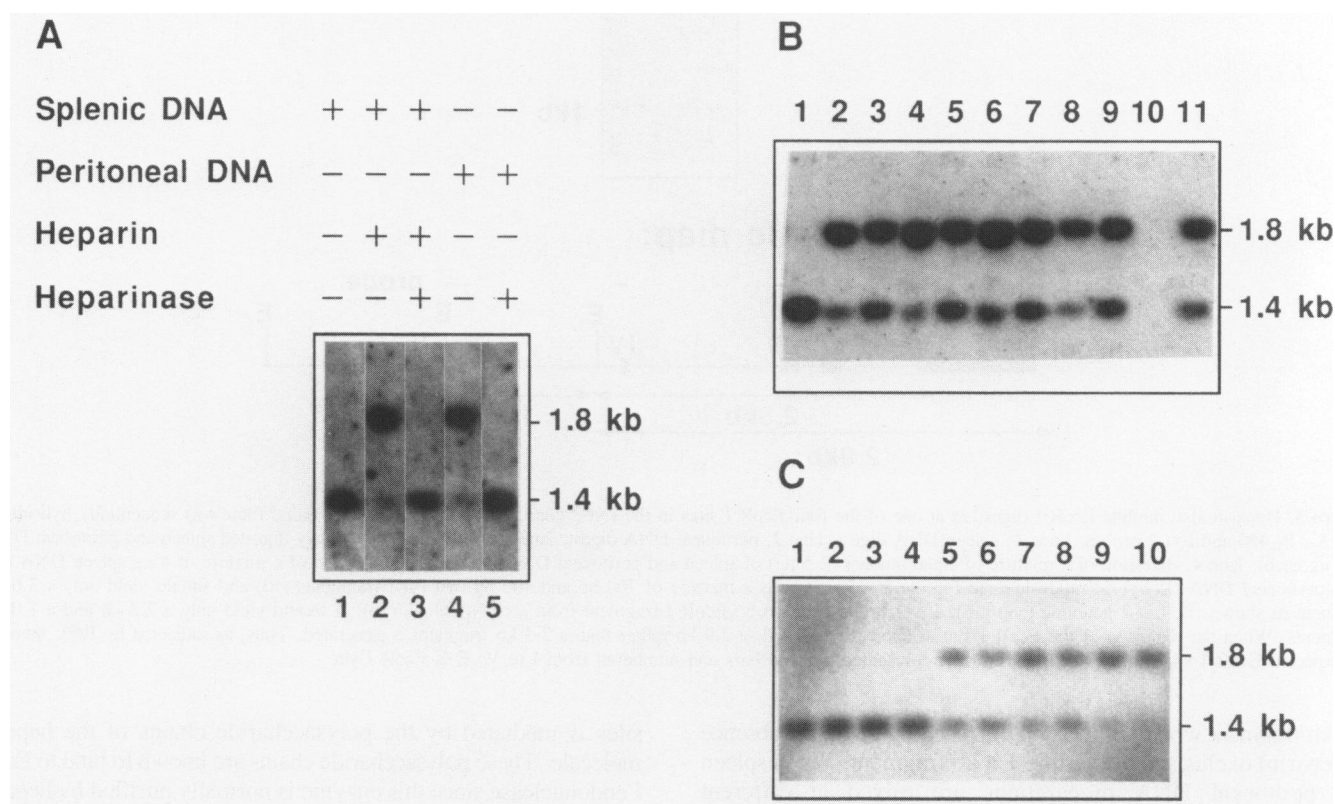
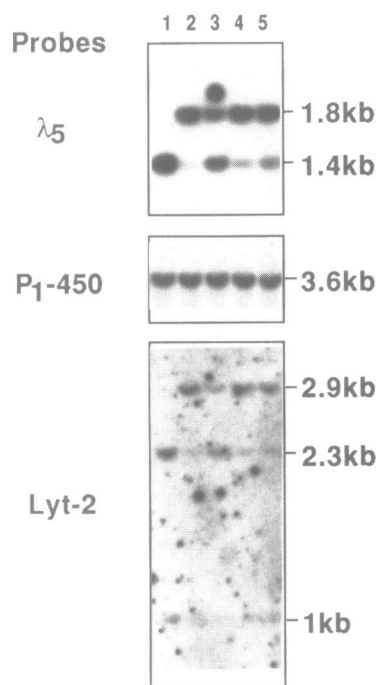


Figure 2. Heparinase treatment abolishes the inhibitory effect. A, purified DNA was treated as below at 37°C overnight, extracted twice with phenol/chloroform/isoamyl alcohol (25/24/1) and twice with chloroform/isoamyl alcohol (24/1), precipitated by ethanol, digested with EcoR I and analysed by Southern as in Fig. 1. Lane 1, spleen DNA with incubation only; lane 2, spleen DNA incubated with 10 μg heparin; lane 3, spleen DNA incubated with 10 μg heparin plus 25 units heparinase; lane 4, peritoneal DNA with incubation only; and lane 5, peritoneal DNA incubated with 25 units heparinase. B, Proteinase K, RNase A and DNase I treatments do not abolish the inhibitory activity. Aliquots of 8 μg peritoneal DNA were treated as below at 37°C for 3 hrs, extracted twice with phenol/chloroform/isoamyl alcohol (25/24/1) and twice with chloroform/isoamyl alcohol (24/1), precipitated by ethanol, and resuspended in 25 μl TE. 20 μl was digested by EcoR I directly (lanes 1, spleen DNA without incubation; lane 2, peritoneal DNA without incubation; lane 3, a mixture of 4 μg spleen DNA and 1 μg peritoneal DNA without incubation; lanes 4, 20 μl of peritoneal DNA with incubation only; lane 5, 5 μg spleen DNA plus 5 μl of peritoneal DNA with incubation only; lanes 6, 20 μl of peritoneal DNA incubated with proteinase K; lane 7, 5 mg spleen DNA plus 5 ml of peritoneal DNA incubated with proteinase K; lanes 8, 20 ml of peritoneal DNA incubated with RNase A; lane 9, 5 mg spleen DNA plus 5 ml of peritoneal DNA incubated with RNase A; lanes 10, 20 ml of peritoneal DNA incubated with DNase I; lane 11, 5 mg spleen DNA plus 5 ml of peritoneal DNA incubated with DNase I. DNase I degrades all DNA as shown by no hybridization in lane 10, however, co-purified heparin remains in the incubation mixture and is able to inhibit spleen DNA digestion as shown in lane 11. C, the inhibitory activity is associated with peritoneal cells. Peritoneal and spleen cells were washed three times separately and then mixed at different proportions. DNA was isolated from each mixture separately and analysed by Southern as in Fig. 1. Lanes 1 and 2 (duplicates), a mixture of 1×10^3 peritoneal cells and 1×10^7 spleen cells; lanes 3 and 4, a mixture of 1×10^4 peritoneal cells and 1×10^7 spleen cells; lanes 5 and 6, a mixture of 1×10^5 peritoneal cells and 1×10^7 spleen cells; lanes 7 and 8, a mixture of 1×10^6 peritoneal cells and 1×10^7 spleen cells; and lanes 9 and 10, a mixture of 5×10^6 peritoneal cells and equal number of spleen cells.

A. Southern blot:



B. Lyt-2 genomic map:

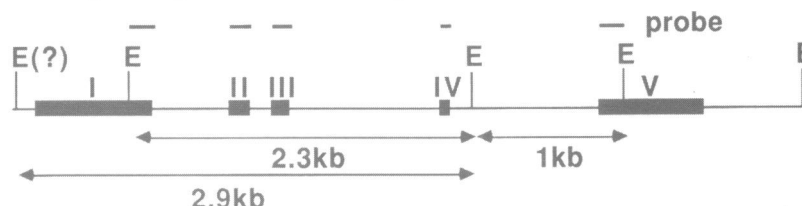


Figure 3. Heparin also inhibits EcoR I digestion at one of the four EcoR I sites in the Lyt-2 gene. A, Southern blot. The same filter was sequentially hybridized with λ_5 , P₁-450 and Lyt-2 probes. Lane 1, spleen DNA digest; lane 2, peritoneal DNA digest; lane 3, a mixture of separately digested spleen and peritoneal DNA (2.5 μ g each); lane 4, digestion of a mixture of equal amount (2.5 μ g) of spleen and peritoneal DNA; and lane 5, digestion of a mixture of 4 μ g spleen DNA and 1 μ g peritoneal DNA. λ_5 hybridization is as in Fig. 1. P₁-450 probe is a mixture of 700 bp and 400 bp two Pst I fragments (15) and should yield only a 3.6 kb fragment as shown. B, Lyt-2 genomic map (16). Lyt-2 probe is a 614 bp EcoR I fragment from a cDNA clone (16). It should yield only a 2.3 kb and a 1.0 kb fragments. When the digestion of the EcoR I site in exon I is blocked, a 2.9 kb rather than a 2.3 kb fragment is generated. Thus, as indicated by E(?), there is an expected EcoR I site 5' of exon I. Exons are represented by bold bars and numbered from I to V. E is EcoR I site.

1.8 kb fragment whereas digestion of spleen DNA (in the absence of heparin) exclusively yields the 1.4 kb fragment. When spleen and peritoneal DNA preparations are mixed at different proportions prior to digestion (Fig. 1, lanes 11 to 14), or spleen and peritoneal cells are mixed at different proportions prior to DNA isolation and digestion (Fig. 2C), the yield of the 1.8 kb fragment is substantially higher than the contribution expected from peritoneal DNA alone. The yield of the 1.4 kb fragment is substantially lower than the complete EcoR I digestion of spleen DNA. These findings show that the co-purified heparin in peritoneal DNA preparation is free and able to complex with EcoR I enzyme and/or EcoR I-DNA complex during digestion.

The commercial heparin used in our experiments is a 13,000 to 15,000 dalton polysaccharide fraction without the core protein as compared to intact heparin of 750,000 daltons in the mouse (3, 4). As we have shown, commercial heparin and the native heparin from mouse peritoneal mast cells have the same inhibitory activity. Thus, heparin inhibition of EcoR I digestion at certain

sites is mediated by the polysaccharide chains of the heparin molecule. These polysaccharide chains are known to bind to EcoR I endonuclease since this enzyme is normally purified by heparin column chromatography (7). Therefore, it is likely that heparin and EcoR I are complexed in the digestion mixture and that this complex is specifically incapable of digesting certain EcoR I sites.

Palindromes probably define the heparin-inhibitable EcoR I sites

We used a series of DNA probes derived from P₁-450, Ly-1, C₁1, DHFR, and Ii genes to search for other EcoR I sites whose digestion might be blocked by heparin. Among these genes, only the EcoR I site in exon I of the Lyt-2 gene is affected resulting in the generation of a 2.9 kb fragment instead of a 2.3 kb fragment expected from complete EcoR I digestion (Fig. 3) (16). Under the same conditions, EcoR I cleavage at this site is inhibited by heparin to the same extent as cleavage of the EcoR I site in the λ_5 exon III. Similarly, digestion of these two EcoR I sites is also

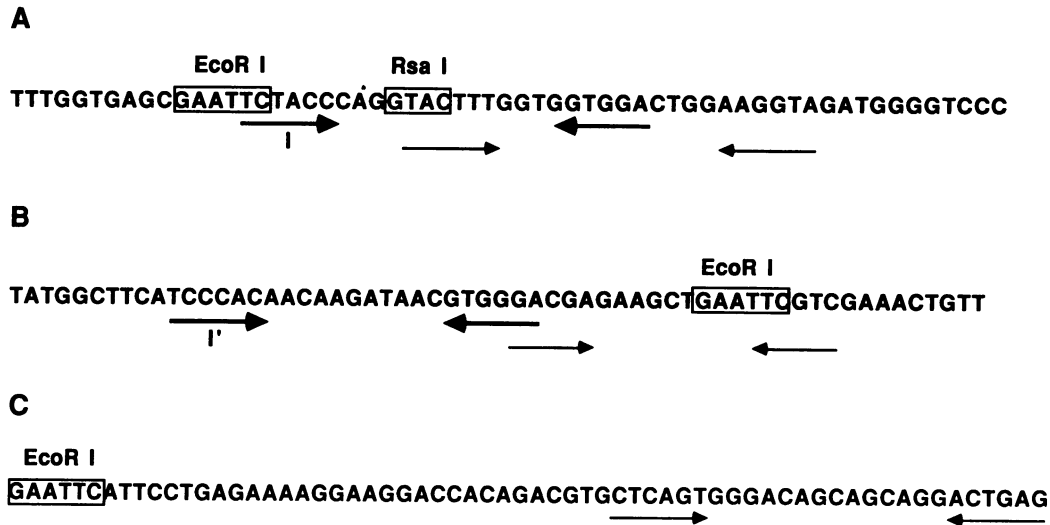


Figure 4. Palindromic sequences are near or overlap with the inhibited EcoR I sites in both the λ_5 and *Lyt-2* genes. The λ_5 gene and the *Lyt-2* gene each contains four EcoR I sites in the known genomic sequences. 100 bp DNA sequences on each side of the eight EcoR I sites are compared (14, 16). A, DNA sequences around the inhibited EcoR I site in the λ_5 exon III. B, DNA sequences around the inhibited EcoR I site in the *Lyt-2* exon I. C, DNA sequences around one of the remaining six uninhibited EcoR I sites. This EcoR I site is thirty-one nucleotide away from the only palindrome found near the remaining six uninhibited EcoR I sites. EcoR I and Rsa I sites are boxed. Palindromic sequences are indicated by arrows. Similar palindromes are labelled as I and I'.

preferentially inhibited over digestion of other EcoR I sites when plasmid DNA p7pB12-1 (14) and pSP-*Lyt-2*, which contain respectively the λ_5 and *Lyt-2* regions of interest, are digested by EcoR I in the presence of heparin (data not shown). Thus, heparin may inhibit cleavage of the two sites by the same mechanism.

The λ_5 gene and the *Lyt-2* gene each contains four EcoR I sites in the known genomic sequences. An examination of 100 bp DNA sequences on each side of the eight EcoR I sites reveals five palindromes (Fig. 4) (14, 16). The inhibited EcoR I site in the λ_5 exon III overlaps with one palindrome and is eight nucleotides away from another. Similarly, the inhibited EcoR I site in the *Lyt-2* exon I overlaps with one palindrome and is nine nucleotides away from another. Furthermore, the palindrome that overlaps with the inhibitable EcoR I site in the λ_5 gene is similar to the palindrome that is nine nucleotides away from the inhibitable EcoR I site in the *Lyt-2* gene, whereas no other homologies are detectable in these regions. In contrast, five of the remaining six EcoR I sites whose digestion are not blocked by heparin have no nearby palindrome. The sixth EcoR I site has one nearby palindrome, however, this palindrome is thirty-one nucleotides away from the EcoR I site. These findings suggest that the location and/or the sequences of the palindromes may affect the digestibility of the neighboring EcoR I sites in the presence of heparin.

DISCUSSION

Studies presented here demonstrate that endogenous heparin copurifies with DNA and inhibits EcoR I endonuclease cleavage of DNA at certain EcoR I sites. Since sufficient amounts of heparin copurifies with DNA, the inhibition results in the appearance of discrete bands that are larger than predicted from genomic maps. Since heparin is widely distributed in animal organs, our findings point out the need for caution when performing restriction fragment length analyses with DNA isolated from cells that are naturally rich in heparin (3, 9). To avoid potential artifacts, DNA isolated from heparin-rich

specimens should be treated with heparinase before EcoR I digestion. Furthermore, commercial heparin is used routinely as an anticoagulant to isolate blood cells and also inhibits EcoR I cleavage of DNA at the same sites. Therefore, we would suggest that anticoagulants other than heparin should be used when isolating DNA from blood cells (8).

The alterations of EcoR I enzymatic activity in the presence of heparin are consistent with other findings demonstrating that EcoR I cleavage rates are influenced by the DNA sequences that flank the EcoR I site. For example, dA·dT base pairs next to the site appear to enhance the rate of cleavage while adjacent dG·dC base pairs slow down the cleavage rate (17–19). Similarly, we find that palindromic sequences near an EcoR I site appear to interfere with digestion of this site when the enzyme is complexed to heparin, either because the complexed enzyme has difficulty recognizing an EcoR I site located near a palindrome or because the presence of the palindrome sharply decreases the rate of cleavage at such a site. In any event, the formation of the heparin-enzyme complex further restricts the specificity of EcoR I endonuclease such that certain sites, particularly those located near palindromes, are no longer digestible by the enzyme.

Since this restricted EcoR I specificity can be achieved by addition of appropriate amounts of heparin to a digestion mixture, the complexed enzyme can be used to produce controlled, partial digests of DNA that contains inhibitable sites. Thus although the introduction of heparin into EcoR I digests should usually be avoided (to prevent artifacts), there are certain circumstances where the addition of heparin to the digestion mixture can prove valuable.

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